Peptide formation on mineral surface under thermal conditions and Stability of combined amino acids under simulated seafloor hydrothermal condition: Implication for the origin of life in sub-seafloor environments

(鉱物表面におけるペプチド生成および熱水条件下における結合体アミノ酸の安定性：海底下における生命発生の可能性)

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Abstract

Seafloor hydrothermal vent, subareal hot springs, deep sediments and volcanic region were proposed as cradles for the life on the primitive earth, however, chemical evolution processes of life precursors are still obscure instead of intense studies. In this study, three different experiments were conducted in order to estimate the favorable conditions for chemical evolution of life on the primitive Earth.

First, the thermal behaviors of glycine (Gly) adsorbed on montmorillonite were observed during heating experiments conducted at 150 °C for 336 h under dry, wet and dry/wet conditions. Approximately 13.9 % of the Gly monomers became peptides on the montmorillonite under dry conditions, with DKP being the most abundant product. Based on results of IR analysis, Gly monomer was mainly adsorbed via H-bonding between positively charged amino group and negatively charged site (i.e., Lewis base site) of montmorillonite surface, suggesting that the Lewis base site acted as a catalyst for peptide formation. On the other hand, peptides were not synthesized in the absence of montmorillonite or on the montmorillonite heated under wet condition. Although a small amount of water from montmorillonite was needed for peptide formation, excess water would promote the hydrolysis of the peptides on montmorillonite under wet condition. Results of this experiments suggested that deep sediments, where dehydration easily occurs, is more appropriate condition to form and condense peptides than the seafloor surface. Moreover, clay minerals, as catalyst of peptide formation, must be essential factors on the primitive earth.

Second, DL-Ala was heated with or without olivine at 120 °C for 8 days, and deastereoisomeric excess of (*de*) Ala-DKP was observed on the surface of olivine. A

small amount of DKP was formed when only DL-Ala is heated. On the other hand, DKP formation was remarkably progressed when heated with olivine. The yield of *cis*-DKP was higher than that of *trans*-DKP because of advantage of a geometric conformation; *cis*-DKP is more thermally stable conformation than *trans*-DKP. The *de* of DKP formed from the reaction without olivine heating for 8 days was +7.3 %, while it was +16.3 % when reacted with olivine. The selective formation of deastereoisomeric peptide, which was enhanced via reaction with olivine, would be important to accumulate homogeneous peptides on the primitive Earth.

Third, seafloor sediment containing much amount of combined amino acids was heated with NaCl solutions for 240 h at 100–200 °C. dissolved combined amino acids (DCAA) and dissolved free amino acids (DFAA) were rapidly released into the solution after starting the reaction, and the concentration of DCAA was consistently higher than that of DFAA. Most of amino acids dissolved in the solution at 100 °C were combined form (90.2 %). The composition of DCAA in the solution heated at 100 °C was very similar with that of THAA in the initial sediment, estimating that biopolymers released from the sediment would be hardly altered during the hydrothermal reaction at about 100 °C or the lower. On the other hand, the ratio of DCAAs to THAA decreased to 71.0 % and 57.7 % at 150 and 200 °C, respectively. In addition, the compositions of DCAA at 150 and 200 °C were significantly different compared with that of THAA in the initial sediment. Thus, low temperature hydrothermal condition (<100 °C) must be required to release and stabilize biopolymers fixed in the sediment without hydrothermal alteration and hydrolysis.

Based on these results, the presumable step of chemical evolution on the primitive Earth was as follows: 1) Amino acids abiotically formed were accumulated in the

seafloor sediment via adsorption mineral onto surface, and then these were oligomerized during diagenesis, 2) homogeneous peptides and proteins were selectively formed on the mineral surface during the oligomerization step, and 3) the biopolymers including peptides and proteins were released from the sediment to ocean by low temperature hydrothermal venting.

This study revealed that 1) adsorption of amino acids onto mineral surface and presence of small amount of water are important for efficiently peptide formation and 2) low temperature hydrothermal conditions (<100 °C) are required to progress chemical evolution on the primitive Earth, and suggested that the possibilities of emergence of life precursor in sub-seafloor environments on the primitive Earth.

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Abbreviations of amino acids

α-amino acids

Ala: Alanine

Arg: Arginine

Asp: Aspartic acid

Cys: Cysteine

Glu: Glutamic acid

Gly: Glycine

His: Histidine

Ile: Isoleucine

Leu: Leucine

Lys: Lysine

Met: Methionine

Phr: Phenylalanine

Pro: Proline

Ser: Serine

Thr: Threonine

Trp: Tryptophan

Tyr: Tyrosine

Val: Valine

Gly peptides

DKP: 2.5-piperazine dione

Gly_2: glycilglycine

Gly_3: glycilglycilglycine

Gly_4: glycilglycilglycilglycine

Gly_5: glycilglycilglycilglycilglycine

Gly_6: glycilglycilglycilglycilglycilglycine

Others

THAA: total hydrolyzable amino acids

DCAA: dissolved combined amino acids

DFAA: dissolved free amino acids

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1. Introduction

Early life was emerged on the primitive Earth at least 3.7 Gyr ago (Otomo et al., 2014). However, the processes of emergence and evolution of life on the early Earth are still undefined. Organic polymers such as proteins, DNA, and RNA are essential precursors to produce the life. Miller and Bada (1988) advocated that the chemical evolution process of life precursor could be divided into three steps:

1) Amino acids and other essential organic compounds were abiotically synthesized and accumulated.

2) The above products were oligomerized to form proteins and nucleotides.

3) Protocells and RNA were formed, and the primitive life was born.

At the present day, two conditions are supposed to synthesize the biomolecules; i.e., endogenous and exogenous. The exogenous condition is explained that the biomolecules produced via cosmic ray radiation in the space were carried with meteorites and/or comet and accumulated on the Earth (Zaia et al., 2008). In fact, small amounts of 80 different amino acids, hydrocarbons, and fatty acids were found in the Murchison (CM2) carbonaceous chondrite meteorites (Kvenvolden et al., 1970; Yuen and Kvenvolden, 1973). It was also revealed that other carbonaceous meteorites such as Allende (CV3), Orgueil (CI1), and Murray (CV2) also contained organic compounds (Sephton et al., 2002). In addition, Observations by infrared, radio, millimeter, and submillimeter frequencies show that a large variety of organic molecules are present in dense and diffuse interstellar clouds (Irvine et al., 1987; Ehrenfreund and Charnley, 2000; Lucas and Liszt, 2000).

The endogenous condition is supposed that the biomolecules were synthesized via

energy emitted by the sun or from seafloor hydrothermal systems. Furukawa et al. (2009) found that numerous organic compounds including fatty acids, amine, and amino acids comprising the terrestrial life were synthesized by shock experiment simulating comet impacts on the Earth' surface.

Although numerous studies reported the abiotic formations of organic compounds in the simulated exogenous and endogenous conditions, the question of origin of life are still a matter of debate. In this study, I discussed possibilities of the chemical evolution, especially amino acids, on the primitive Earth were discussed based on some experimental results.

1.1 Amino acid, peptides and proteins

Amino acids, peptides, and proteins are ones of the essential organic compounds to construct life bodies. Amino acids are organic compounds composed of amino ($-NH_2$) and carboxyl ($-COOH$) groups and have two enantiomers: L-form and D-form (Fig. 1-1). They are classified according to types of side-chain ($-R$ group), and 20 types of amino acids including asparagine and glutamine are used for the formation of terrestrial life bodies (Table 1-1). Peptides and proteins are formed through oligomerization of the amino acids. The oligomerization of amino acids, i.e., peptide formation (n amino acids $\rightarrow$ peptide + $n-1$ H_2O), is essential to product primitive proteins. Therefore, it is of interest to determine the physico-chemical conditions that form and stabilize amino acids, peptides and proteins to constrain conditions where life was born on the primitive Earth.

1.1.1 Abiotic synthesis of amino acids on the primitive Earth

Possibilities of the amino acid synthesis on primitive Earth have been investigated through various experiments since Miller (1953) had successfully synthesized some amino acids (Gly, Ala, and Asp) under a simulated reducing atmosphere (CH_4, H_2O, NH_3, and H_2). Sugan and Khara (1971) observed formation of Gly, Ala, Cys, Asp, and Glu in the reducing atmosphere composed by CH_4, C_2H_6, NH_3, H_2O and H_2S when ultraviolet light was used as energy source. Ruiz-Bermejo et al. (2007) synthesized protein amino acids (Gly, Ala, Pro, Ser, Cys, and Asp) and non-protein amino acids during a spark discharge experiment, which were designed by both the reducing atmosphere (CH_4, H_2O, and H_2) and saline solution containing FeS.

However, the atmosphere of the early Earth would have been rather oxidizing evidenced by the presence of CO, CO_2, and N_2 than the previously assumed (Kasting and Ono, 2006; Zaia et al., 2008). Kobayashi et al. (1990) observed that some α-amino acids (Asp, Thr, Ser, Gly, and Ala) were synthesized when a mixture of CO and N_2 was irradiated by high energy protons (3 MeV, 0.6 μA). They also found that concentration of the amino acids was higher in the atmosphere where CO and N_2 were stable than reducing atmosphere where CH_4 and N_2 were present. Janda et al. (2008) synthesized Gly, Pro and Val in the atmosphere composed of CO_2, N_2, and H_2O via electron emission and estimated that the production of CO through the decomposition of CO_2 was important for the further synthesis of organic compounds.

It was also revealed that α-amino acids were abiotically synthesized under simulated hydrothermal conditions. The hydrothermal environment is postulated to have been the cradle of life on the primitive Earth (e.g., Corliss et al., 1979; Miller and Bada, 1988). Hennet et al. (1992) observed formations of Asp, Ser, Glu, Gly, Ala, and Ile in a hydrothermal solution dissolving KCN, NH_4Cl, and HCHO with coexisting illite,

titanium oxide, and platinum after heating at 150 °C for 54 h. When a solution containing 0.1 M ammonium formate (NH_4HCO_2) was heated under simulated hydrothermal conditions of 50–292 °C and 150 atm for 20 min through a flow reactor, Gly was formed as the dominant amino acid (Aubrey et al., 2009). However, these experimental settings are unrealistic, because they required high starting concentration of reduced reactive compounds such as NH_4HCO_2, KCN, and HCHO (Aubrey et al., 2009). Kobayashi et al. (1995) found the formation of some amino acids (Asp, Ser, Glu, Gly, and Ala) under more reasonable hydrothermal conditions, which contained CO_2, CH_4, NH_4Cl, and some metals (Fe, Mn, Zn, Cu, Ca, Ba), and concluded that the metal ions would catalyze the synthesis of organic compounds.

1.1.2 Peptide formation experiments

The peptide formation is endothermic dehydration reaction (Shock, 1992). Gibbs free energy is 28.3 kJ/mol when Gly changes to Gly_2 (2 Gly → Gly_2 + H_2O) at room temperature and 1 bar (Dick et al., 2006; Kitadai et al., 2011), indicating that the peptide formation reaction is unfavorable at the present Earth's surface. However, the free energy decreases to 16.0, 7.9, and 2.9 kJ/mol at 160, 220, and 260 °C, respectively (Lemke et al., 2009). Thus, high temperature condition is required to form the peptide bonds.

Some researchers found that protein-like polymers (proteinoids) are synthesized through melting of an anhydrous mixture of acidic and basic amino acids, such as Asp, Glu, and Lys, at high temperature (120–200 °C) (Harada and Fox, 1960; Fox and Nakashima, 1967; Melius and Srisomsap, 1997). However, the proteinoids contain were combined by only a small amount of peptide bonds but mainly by ester-like bonds

(Andini et al., 1975; Fitz et al., 2007). Polymer balls of amino acids (0.3−2.5 μm) called marigranules were observed when amino acids were heated to 105 °C in an artificial hydrothermal solution containing dissolved Mg, Ca, Mo, Zn, Fe, Cu, Mn, and Co (Yanagawa and Egami, 1978). Short chained peptides were synthesized by heating solution containing highly concentrated amino acids in a flow reactor, which simulates the rapid heating and cooling conditions of hydrothermal vents (Imai et al., 1999; Cleaves et al., 2009). However, the formation rates were very low (<1%) and the peptides were hydrolyzed by rapid hydrolysis (Qian et al., 1993; Bada et al., 1995). Thus, catalysts are necessary to yield enough peptides to survive from the hydration.

1.1.3 Effects of catalysis on peptide formation

Inorganic salts and metal ions effectively work as catalysts to form oligopeptide, and this process is called salt induced peptide formation (SIPF) hypothesis (Rode, 1999). Schwendinger and Rode (1989) heated the solution dissolving 0.4−0.8 M Gly and 0.5−5 M with sodium chloride at 85 °C, and Gly_2 were synthesized only when $CuCl_2$ was added in the solution, indicating that the peptide formation must be catalyzed by copper ion. The concentration of copper ion was important factor for the SIPF. When the molar ratio of (amino acids):(copper ion) is 2:1, peptide concentration were the highest (Eder and Rode, 1994), probably because two amino acid molecules complexed with a copper ion (Napier and Yin, 2006). The complex was formed as below (Schwendinger and Rode, 1992; Eder and Rode, 1994): 1) coordinate bond was formed between an amino acid and copper ion, and complexed with chloride ions. 2) Another amino acid bound to this complex via its carbonyl group; there were two amino acids in the copper complex. 3) The nitrogen atom of chelating amino acid attacked the carbonyl carbon of the other

amino acid, and a peptide bond is formed. Longer peptides are also formed through the same process repeated (Suwannachot and Rode, 1998). Sakata et al. (2014) evaluated the effects of metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, and Cu^{2+}) on peptide formation rates under a simulated hydrothermal condition. Gly_3 was synthesized only when copper ion was dissolved, confirming that the formation of copper complex was important to elongate the peptides.

Minerals are also efficient catalysts for peptide formation reactions (Lambert, 2008). The behavior of amino acids on mineral surfaces has been investigated since the possibility of peptide formation on clay mineral surfaces was proposed by Bernal (1951). Clay minerals are good candidates for the production of the biopolymer because of the strong affinity of amino acids adsorption on the high specific surface area (Greenland et al., 1964; Hedges and Hare, 1987; Benetoli et al., 2007). Some researchers have observed short peptide formations on clay minerals in dry−wet cycle experiments (Lahav et al., 1978; Bujdak and Rode, 1996; Ferris et al., 1996; Son et al., 1998; Bujdak and Rode, 1999a; Brack, 2013). They found that peptide formation was promoted by the adsorption of amino acids on clay mineral surfaces and particle edges. However, the mechanisms for specific peptide formation from adsorbed amino acids are unclear because the adsorption conditions of amino acids on clay minerals cannot be directly determined.

1.1.4 Racemic problems

Asymmetric stereoisomer of proteins is also one of puzzling questions concerning origin of life. As described above, biological proteins are mainly composed L-form amino acids. However, equivalent rate of enantiomers appears when amino acids are

abiotically synthesized. Therefore, it is important to estimate the physico-chemical factors that produce enantiomeric excess (*ee*) of amino acids to form protein precursors on the primitive Earth.

The main hypothesis explains that the selective formation and accumulation of L-amino acids were firstly important for the formation process of protein precursors. The *ee* of amino acids was observed in a Murchison meteorite; L-Ala and L-Val were 1.2 % and 2.2 % exceeded respectively (Cronin and Pizzarello, 1997). Meierhenrich et al. (2005), suggesting that the *ee* of Leu in solid state was produced by vaccum ultraviolet asymmetric photochemical reactions, such as enantioselective photolysis, under interstellar/circumstellar conditions. Takano et al. (2007) found the asymmetric amino acid precursors were synthesized when a gas mixture (CO, NH_3, and H_2O) was irradiated with 3.0 MeV protons.

Some researchers estimated that the *ee* proceeded during peptide formation (Morowitz, 1969; Blackmond, 2011). However, the peptide formation is promoted at high temperature conditions as described above. The *ee* of amino acids would disappear due to racemization at high temperature condition.

1.2 Stabilities of amino acids, peptides and proteins under hydrothermal conditions

The stability and behavior of the biomolecules have been searched in various conditions of the earth. As described above, abiotic formations of amino acids and peptides are promoted under high temperature condition. However, biomolecules including amino acid, peptides and proteins become labile with increasing temperature, and that they do not exist in significant concentrations at temperatures over 200 °C (Bernhardt et al., 1984; White, 1984).

The stability of amino acids and peptides were mainly studied under simulated hydrothermal conditions. When biological protein isolated from Pyrodictum occultum was heated under a hydrothermal condition (250 °C, 260 atm), it was completely hydrolyzed only 6 h (Bernhardt et al., 1984). Miller and Bada (1988) evaluated the stabilities of individual amino acids (Asp, Leu, and Ser) under a simulated hydrothermal condition (250 °C, 265 atm) and observed that most of these amino acids were decomposed for only 5 h. Qian et al. (1993) revealed that the amino acids were rapidly degraded according to first order kinetics, and indicated that amino acids rarely survived under hydrothermal conditions >200 °C for long periods of time.

In contrast to most of the hydrothermal laboratory experiments, which are performed in closed systems (batch type reactors), natural hydrothermal systems are open and occasionally at a non-equilibrium state (Imai et al., 1999). Flow type reactors can produce rapid heating-cooling and supercritical conditions (e.g., Islam et al., 2003). The flow type experiments can simulate natural conditions more closely than can batch type experiments. When Gly solution was heated in a flow reactor for 10 sec, much of the Gly remained even when the solution reached >350 °C and 40 MPa (Alargov et al., 2002). This finding indicates that amino acids can survive in an instantaneous supercritical condition.

1.3 Amino acid in natural hydrothermal samples

Several studies reported that samples recovered from natural hydrothermal systems including fluids, sediments, and rocks contained high amount of amino acids. Haberstroh and Karl (1989) determined the concentration of dissolved free amino acids (DFAAs) in hydrothermal fluids and pore waters of sediments collected from the

Guaymas Basin in the East Pacific Rise, where beneath 500 m of sediment, hydrothermal systems actively discharged high temperature fluids >300 °C. Small amount of DFAAs were detected (<1 nM) in the hydrothermal fluids. The sediment pore water contained 5–445 μM DFAAs, with glutamic acid, glycine, serine and alanine being the most abundant amino acids. Andersson et al. (2000) measured the concentrations of amino acids in the hydrothermally altered sediments cored from the Juan de Fuca Ridge in the northeastern Pacific Ocean; the total hydrolysable amino acids (THAA) in their samples was 100–2000 nmol/g, with glycine, alanine, serine, and histidine as the major components. Takano et al. (2005) studied amino acids in solid samples taken from the seafloor around a hydrothermal vent at Suiyo Seamount in the western Pacific Ocean; the concentrations of THAA in the cored rocks and the chimneys were 26–107 nmol/g and 11–64 nmol/g, respectively. Hydrothermal fluids >300 °C collected from the Suiyo Seamount hydrothermal vents contained 246–1163 nM THAA, with abundant glycine and serine (Horiuchi et al., 2004). Highly concentrated amino acids were detected from the low temperature hydrothermal fluids (42–89 °C) collected at Vulcano Island in Italy, and the highest amino acid concentrations were found at sites with relatively-high temperature (82 °C), acidic (pH 2.0) and reducing conditions (Svensson et al., 2004). Fuchida et al. (2014) found that concentrations of AAs, most of which were polymer form, were 4–54 times higer in high temperature fluids (>208 °C) than in low temperature fluids (<53 °C) and ambient seawater collected from Mariana Trough (Fig. 1-2). These observations supported that biological amino acids, peptides and protein could survive at high temperature conditions, and indicated that natural hydrothermal fluids are a pool of organic compounds.

1.4 A new hypothesis for chemical evolution and purpose of this study

Furukawa et al. (2009) found that various aqueous organic compounds including amino acids were synthesized by impact-shock on water phase. Oxygen isotopic compositions of the Hadean zircon crystals indicated that primitive ocean existed at least 4.4 Gyr ago (Mojzsis et al., 2001; Wilde et al., 2001) In addition, it was estimated that heavy bombardment by extraterrestrial meteorites occurred caused 3.9–4.0 Gyr (Fegley et al., 1986). Therefore, Nakazawa and coworkers suggested that organic compounds were synthesized in the atmosphere and condensed in the Hadean ocean during the heavy bombardment (they called “the big bang of organic molecules”) as first step of the chemical evolution. The organic compounds must be survived in the seafloor sediment at high concentrations, since these did not experience ultraviolet degradation, biotic utilization, and oxidation (Furukawa et al., 2012). As second, organic monomers such as amino acids were oligomerized and biopolymers such as proteins were accumulated in the deep seafloor sediment by geothermal heating and dehydration during diagenesis (Nakazawa, 2005; Furukawa et al., 2012). Some studies revealed the oligomerization of amino acids without catalysts under high temperature and pressure conditions simulated the deep seafloor environments (Ohara et la., 2007; Otake et al., 2011; Furukawa et al., 2012). Finally, the biopolymers would be released from sediment to ocean by hydrothermal fluid flows and life precursors were formed and evolved in the ocean (Fig.1-2) (Nakazawa, 2008).

Nakazawa and coworkers assumed that amino acids were accumulated and oligomerized in seafloor sediment via adsorption on clay minerals. Seafloor sediments contained much amount of clay minerals, and these must have worked as efficient catalysts for oligomerization. However, the mechanisms of peptide formation from

adsorbed amino acids are still unclear because the adsorption conditions of amino acids on clay minerals cannot be directly determined.

In addition, the hydrothermal release process of biopolymers fixed in the seafloor sediment was also still unclear. Ito et al. (2006) and Yamaoka et al. (2007) heated the seafloor sediments including abundant biological AAs with NaCl solution to simulate natural seafloor hydrothermal conditions and evaluate stabilities of AAs in the fluid. However, these studies did not reveal the stability of proteins (i.e., combined amino acids) in the hydrothermal condition, since they analyzed only concentration of THAA.

Thus, in this study, three deferent experiments were designed to clear the peptide formation mechanisms on minerals under dehydration conditions and stability of combined form amino acids under hydrothermal conditions:

1) Gly-montmorillonite was heated at 150 °C for up to 336 h under dry and wet conditions.

As shown above, some researchers have observed peptide formation on clay mineral surface. However, the mechanism of peptide formation on the clay mineral surface under dehydration conditions has not been clarified. In this study, the thermal behavior of Gly adsorbed on montmorillonite was observed during heating experiments conducted at 150 °C for 336 h under dry, wet, and dry–wet. Montmorillonite is a smectite group mineral that has a 2:1 layer structure with a high surface area and a high exchangeable cation capacity. Gly is the simplest neutral amino acid ($H_2N–CH_2–COOH$) and is achiral. Gly is the most abundantly produced prebiotic amino acid (Miller 1953; Kobayashi et al. 1990; Zaia et al. 2008) and has high reactivity (Schwendinger and Rode 1992; Suwannachot and Rode 1998). The roles of water and

clay minerals in peptide formation were evaluated based on the experimental results, and possible conditions where peptide formation first occurred on Earth are proposed in relation to the chemical evolution of primitive life.

2) Diastereoisomers of Ala-DKP was synthesized on olivine surface.

As previously described, asymmetric stereoisomer of proteins is also problem concerning origin of life. I hypothesized that the *ee* was produced on mineral surfaces during oligomerization.

It was suggested that peptide has two diastereomers, i.e., *cis* and *trans* forms (Nagayama et al., 1990) and the *cis* form peptides has a homogeneous conformation. Thus, the excess formation of the *cis* peptides would lead to the *ee* of amino acids in proteins.

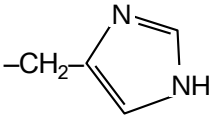
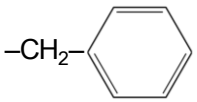
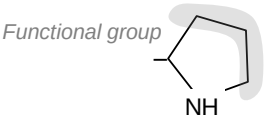
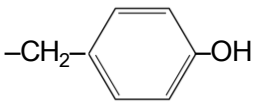
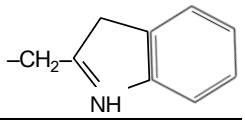
When peptides are abiotically synthesized from amino acid monomers under thermal condition, cyclic dipeptides (DKP), are intensely formed. Diastereoisomeric DKP (*cis*/*trans*) is formed, when chiral amino acids are oligomerized (Fig. 1-3). In this study, the deastereoisomeric excess (*de*) of DKP formed from the simplest chiral amino acid, i.e., Ala, was observed on the surface of olivine, which was heated at 120 °C for 8 days.

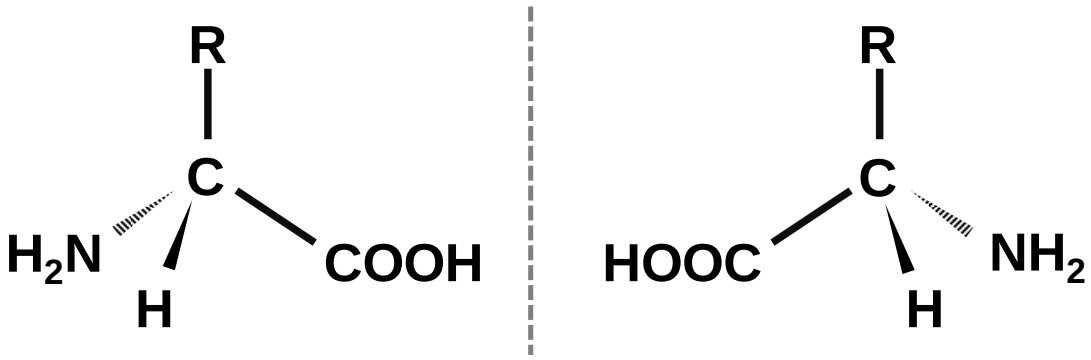
3) Natural seafloor sediment containing biopolymers were heated under simulated hydrothermal conditions.

It was revealed that biopolymers including polypeptides and proteins were labile and hydrolyzed under hydrothermal conditions (Miller and Bada, 1988). If the biopolymers were released from sediment to ocean via hydrothermal fluid flows, these need to survive under the high temperature conditions. Thus, natural seafloor sediments

containing much amount of combined form amino acids were heated with NaCl solutions at 100–200 °C for 240 h and dissolved combined amino acids (DCAA) and dissolved amino acids (DFAA) in hydrothermal solutions were determined in order to evaluate the stabilities and observe the release and pyrolysis process of biological DCAA.

Table 1-1 Side chains of protein type α-amino acids

Acidic amino acids		*Neutral amino acids*	
Asp	$-CH_2COOH$	Gly	$-H$
Glu	$-CH_2CH_2COOH$	Ala	$-CH_3$
Basic amino acids		Ser	$-CH_2OH$
Lys	$-CH_2CH_2CH_2CH_2NH_2$	Cys	$-CH_2SH$
Arg	$-(CH_2)_3NHC(NH_2)=NH$	Val	$-CH(CH_3)_2$
His		Thr	$-CH_2(OH)CH_3$
		Met	$-CH_2CH_2SCH_3$
		Leu	$-CH_2CH_2(CH_3)_2$
Aromatic amino acids		Ile	$-CH_2(CH_3)CH_2CH_3$
Phe		Pro	
Tyr		Trp	



L-form amino acid D-form amino acid

Fig. 1-1 Mirror model of racemic amino acid molecules

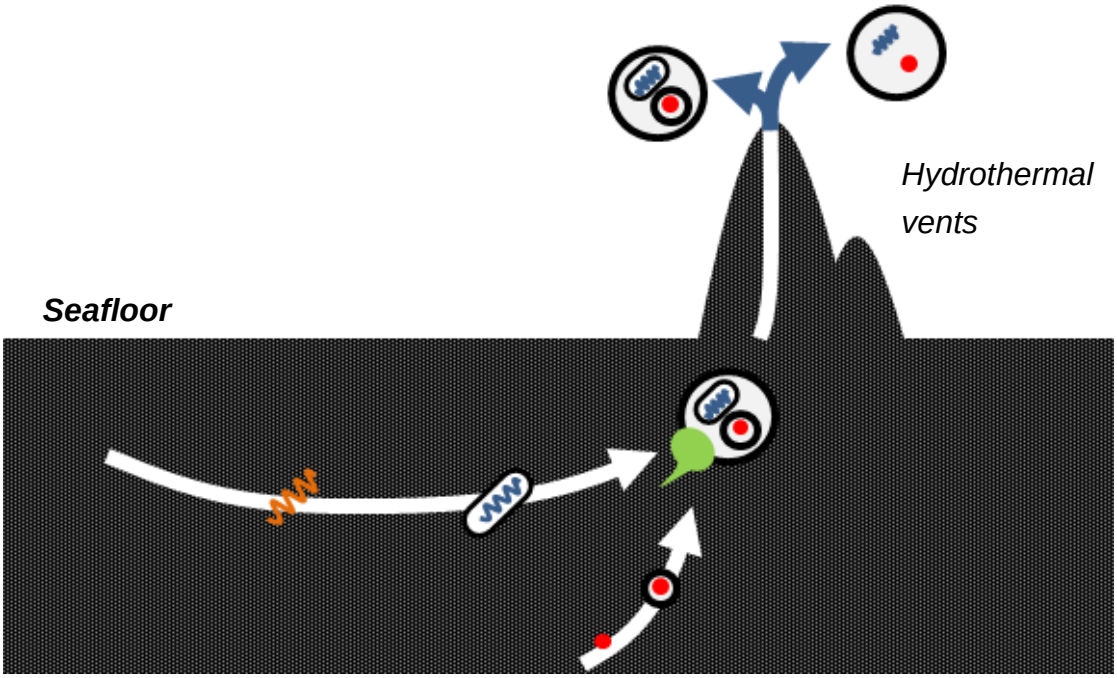
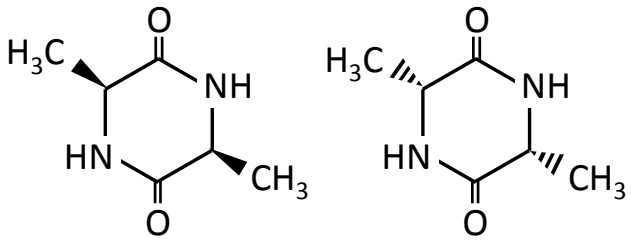


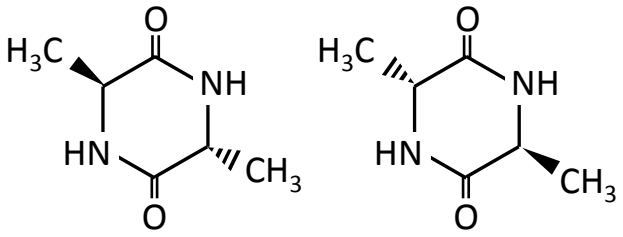
Fig. 1-2 Model of release of biopolymers and life precursors from sediment to ocean by hydrothermal fluid flows (Nakazawa, 2014)

***cis*- DKP**



L+L *D+D*

***trans*- DKP**



L+D *L+D*

Fig. 1-3 Diastereoisomers of Ala-DKP

2. Experimental

2.1 Materials

2.1.1 Chemicals

Amino acids (pure grade: 99 %), Gly_2 (pure grade; 98 %), Gly_3 (pure grade: 99 %), 2.5-piperazine dione (DKP, pure grade: 99 %), DL-Ala anhydride (Ala-DKP, pure grade: 97 %), and other reagents for high-performance liquid chromatograph (HPLC) (analytical grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.1.2 Montmorillonite

Montmorillonite was purchased from The Clay Science Society of Japan (Okayama, Japan). The composition of the montmorillonite was $(Na_{0.90}K_{0.37}Ca_{0.13})[(Fe_{0.30}Al_{2.77}Mg_{0.93})(Al_{0.55}Si_{7.17})O_{20}](OH)_4 \cdot nH_2O$, and small amounts of quartz and cristobalite were present as impurities based on the result of X-ray fluorescence analysis. The cation exchange capacity (CEC) of the montmorillonite was 86.4 meq/100g, and the Brunauer–Emmett–Teller surface area was estimated to be 27.35 m^2/g (Miyawaki et al., 2010).

Before the experiments, the montmorillonite was reacted with 1M NaCl solution to saturate in the exchangeable layer with Na^+, and washed repeatedly with ultrapure water to remove dissolved salts in the reacted solution. To check the complete removal of dissolved salts, chloride ion was tested using $AgNO_3$.

2.1.3 Seafloor sediment

Seafloor sediment used for hydrothermal experiment was collected at Izena Caldron, Okinawa Trough (27°14.56'N, 127°03.97'E) (Fig. 2-1) during KT 10-22 cruse (15–21 Oct. 2010, R/V Tanseimaru), and preserved in a deep freezer (–80 °C). In the laboratory, the freezed sediment samples was dried in a vacuum, powdered manually with agate mortar, and sieved with a 48 mesh.

2.2 Clay mineral experiment

To adsorb Gly on montmorillonite, 3.0 g of the prepared Na-montmorillonite sample was stirred into 30 mL of 0.1 or 1 M Gly solution in a polytetrafluoroethylene (PTFE) centrifuge tube (50 cm^3) and then the tube was shaken at room temperature for 2 h. The solid was separated by centrifugation (12000 rpm for 20 min), washed twice with 30 mL of ultrapure water, and dried in a vacuum. Hereafter, the Gly treated montmorillonite is called Gly-montmorillonite.

Approximately 0.1 mg Gly-montmorillonite was placed in a 2 cm^3 glass ampoule. Ultrapure water (0.1 mL) was added to some samples, and the ampoule was flushed with Ar and sealed. Hereafter, the conditions with and without the addition of ultrapure water are referred to the wet and dry conditions, respectively. It should be noted that 4–5 wt.% water was retained Gly-montmorillonite after vacuum drying. Thus, some water was present in the system even under the dry conditions. The prepared ampoules were kept in a drying oven at 150 °C for 336 h. For the dry−wet experiments, the montmorillonite was heated in the ampoule without additional water for 168 h in a drying oven at 150 °C. Then, 0.1 mL water was added in the ampoule and it was sealed and heated again for 168 h under the same conditions (total heating time was 336 h). Five samples were prepared for each set of conditions.

After heating, the samples were freeze-dried and the reactant was replaced with 4 mL of a 0.1 M $CaCl_2$ solution in a 5 cm^3 glass ampoule. The ampoule was sealed in Ar atmosphere and left at room temperature for 24 h to desorb Gly and its peptides from the montmorillonite. This procedure is frequently used to desorb amino acids and peptides adsorbed on clay minerals (Lambert, 2008). Bujdak and Rode (1999b) tested the efficiency of $CaCl_2$ solution to detach adsorbed amino acids and peptides. They demonstrated that the difference between the concentrations of the added and desorbed amino acids was not significant, and IR peaks derived from the remaining amino acids on the clay mineral surface were not detected after the treatment with $CaCl_2$ solution. The reacted $CaCl_2$ solution was filtered through a PTFE membrane filter (0.2 μm) using a disposable syringe. The desorbed Gly monomer and peptides were quantified using a high performance liquid chromatograph (HPLC). The error associated with the desorption procedure was within ± 0.4%.

2.3 Olivine experiment

Olivine (San Carlos, AZ, USA) was manually powdered with agate mortar. DL-Ala powder was mixed with/without powdered olivine and a small amount of ultrapure water in a 2 cm^3 glass ampoule, which was sealed under Ar atmosphere. The ampoules were heated in a drying oven at 120 °C for 1–8 days. After cooling at room temperature, the reacted product was suspended in 5 mL ultrapure water and the dissolved diastereoisomers of DKP were quantified using the HPLC.

2.4 Hydrothermal experiment

1.0 g of powdered sediment was reacted with 15 mL of 3.5 % NaCl in a tightly closed

PTFE vessel (25 cm^3) contained in a stainless steel vessel. The vessel was closed after flushing Ar and kept in a drying oven at 100, 150, and 200 °C for 240 h. After heating, reactant transferred into a PTFE centrifuge tube (50 cm^3) and then solid phase was separated by centrifugation (12000 rpm for 10 min). The reacted solution was filtered through a PTFE membrane filter (0.45 μm) and stored at –20 °C. The separated solids were dried in a vacuum, powdered manually with agate mortar, and stored at –20 °C.

The solution and dried sediment were hydrolyzed to extract the amino acids from the hydrolyzable peptides and proteins following a previously published procedure (Kawahata and Ishizuka, 1993; Andersson et al., 2000; Ito et al., 2009). Approximately 3 mL of liquid sample was put into a glass ampoule (10 cm^3) with 3 mL of concentrated 12 N HCl. In case of sediment sample, 0.5 g of the dried sediment was put in the glass ampoule with 4 mL of 6 N HCl. Air was flushed out with Ar, and the ampoule was sealed and heated in a drying oven at 110 °C for 22 h. After cooling to room temperature, the solution was filtered through a PTFE membrane filter (0.2 μm) using a disposable syringe and transferred into a pear-shaped flask. The unreacted HCl in the solution was removed by evaporation under vacuum at 40°C, and the residue was dissolved in ultrapure water.

2.5 HPLC analysis

2.5.1 Quanrification of α-amino acids

The amounts of α-amino acids including Gly were quantified by HPLC using postcolumn ortho-phthalaldehyde derivation (Benson and Hare, 1975). Fifteen types of α-amino acids: Asp, Thr, Ser, Glu, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, Lys, His, and Arg were separated through a cation exchange resin (Hitachi, #2619PH, 4.0×150 mm

i.d.). The eluent was a citrate buffer solution (3-sodium citrate, citric acid, sodium chloride, and ethanol), and the flow rate was 0.4 mL/min at 60 °C. The separated amino acid were derivatized by boric acid buffer solution containing 6 mM ortho-phthalaldehyde, and the amino acid derivatives were detected by a GL-7453 fluorometric detector (GL Science Inc., Tokyo, Japan) (excitation wavelength: 360 nm; emission wavelength: 440 nm). Figure 2-2 shows examples of the chromatograms of standard solution, which contains each 50 μM amino acid. Detection limits of each amino acid were less than 10 nM in this analysis.

2.5.2 Quanrification of Gly peptides

The Gly peptides were separated using a GL Sciences InertSustain C18 column (length: 4.6×250 mm), and analyzed by a UV detector (GL-7450, GL Sciecnce) at 195 nm absorbance. The eluent was a mixture of 10 mM KH_2PO_4 and 7.2 mM $C_6H_{13}SO_3Na$, adjusted to pH 2.5 using H_3PO_4. The flow rate of the mobile phase was 1.0 mL/min at 40 °C. Figure 2-3 shows examples of the chromatograms of standard.

2.5.3 Separation of DKP diastereoisomers

The *cis* and *trans* Ala-DKP were separated using the GL Science Inertsustain C18 column (length: 4.6×250 mm), and analyzed by the UV detector (GL-7450) at 195 nm absorbance. The eluent was MeOH/H_2O (96/4, v/v), and the flow rate of the mobile phase was 1.0 mL/min at 40 °C. Figure 2-4 shows an example of the chromatograms of standard solution.

2.4 XRD and FT-IR analysis

2.4.1 X-Ray Diffraction

X-ray diffraction (XRD) (Rigaku Geigerflex RAD-IA, Tokyo, Japan) was carried out to measure the difference of the basal layer (001) spacing of the Gly-montmorillonite and the others, using Ni-filtered monochromatic CuKα radiation with 2θ angle between 2° and 14°.

2.4.2 *Fourier Transform Infrared Spectroscopy*

The infrared (IR) spectra were obtained using a FT-IR 8100 spectrometer (Shimadzu Corp., Kyoto, Japan). For the IR spectroscopy, a mixture of 2 mg sample and 100 mg KBr was pressed to make an optically transparent pellet. The pellets were scanned 95 times at a spectral resolution of 8 cm^{-1} from 400 to 4000 cm^{-1}.

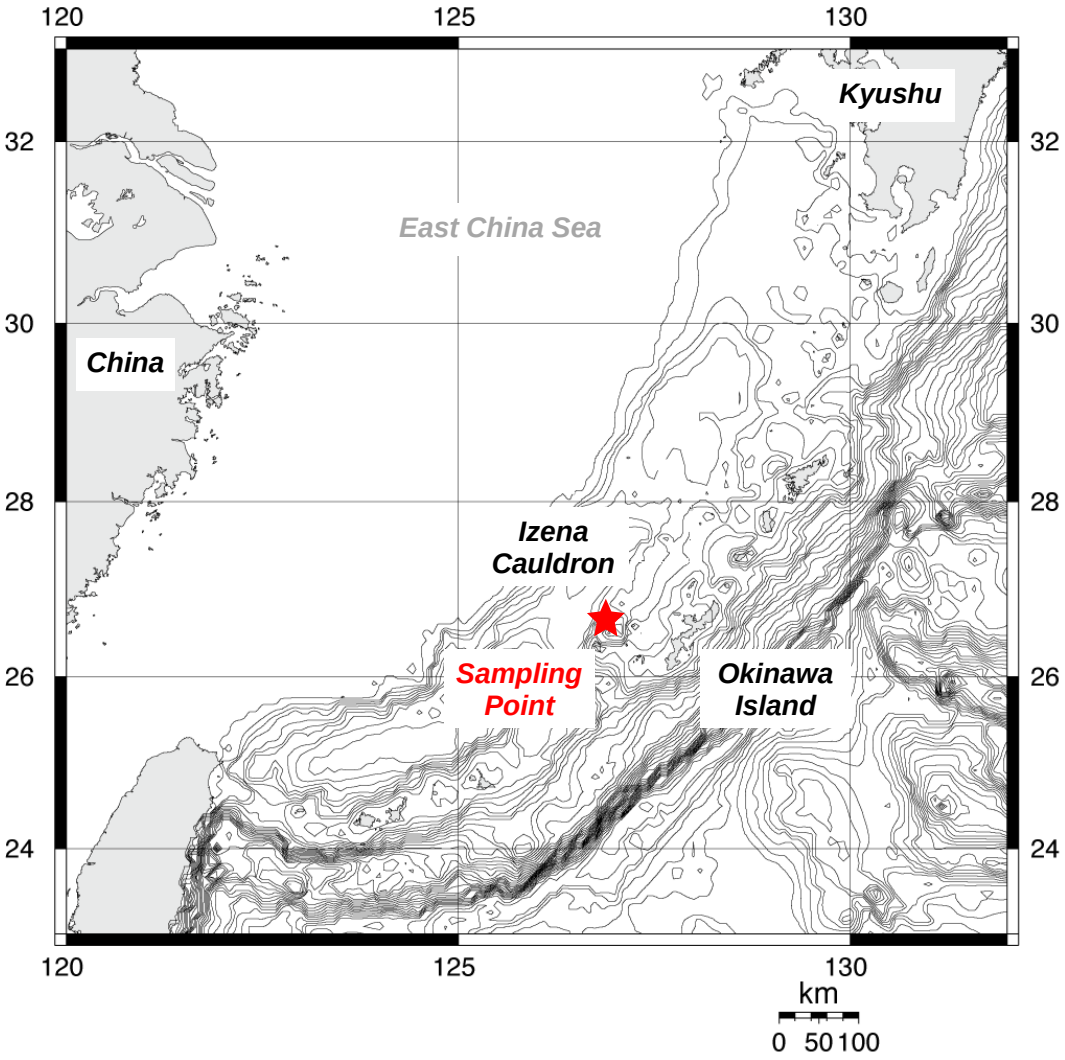


Fig. 2-1 Sampling point of seafloor sediment

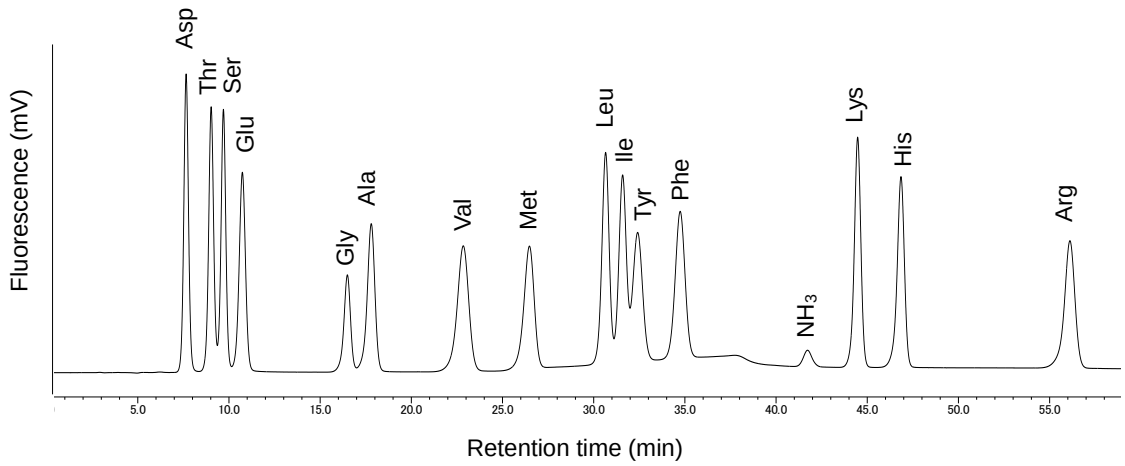


Fig. 2-2 Chromatogram of standard solution contained α-amino acids (50 μM)

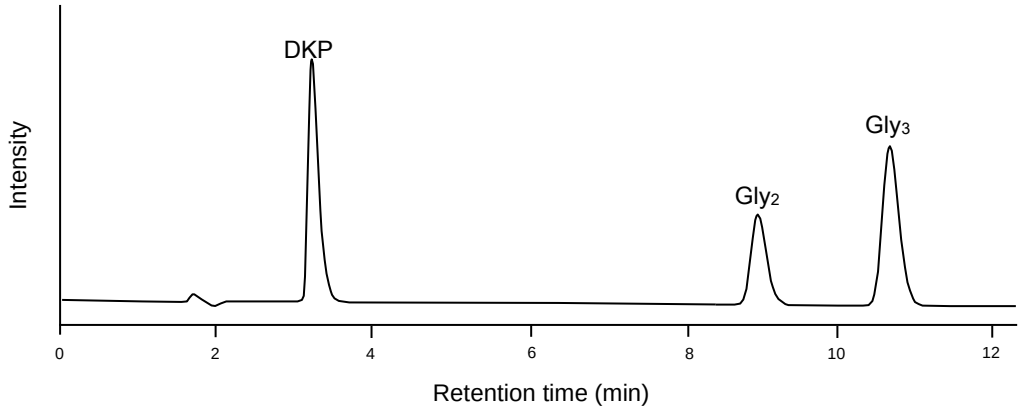


Fig. 2-3 Chromatogram of standard solution contained DKP, Gly_2 and Gly_3 (500 μM)

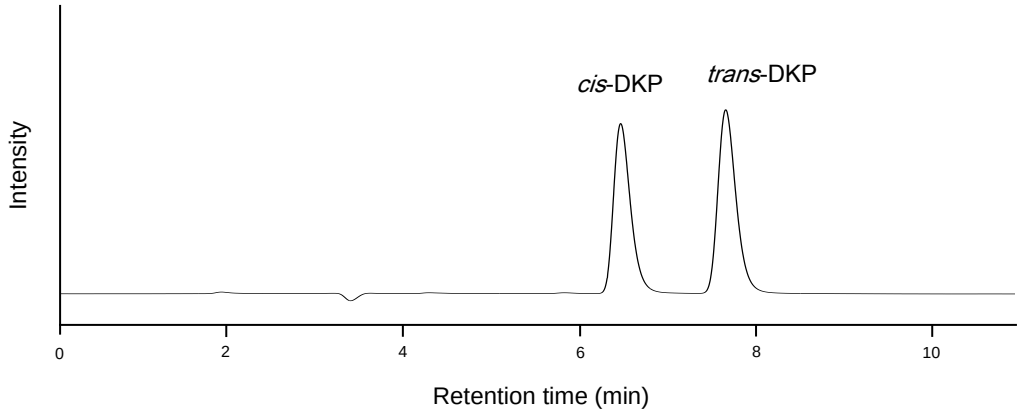


Fig. 2-4 Chromatogram of standard solution contained *cis*- and *trans*-DKP (500 μM)

3. Result

3.1 Clay mineral experiment

3.1.1 Changes in Gly monomer concentration on montmorillonite

The amount of Gly adsorbed on the montmorillonite was 234.7 μmol/g when the montmorillonite was treated with 0.1 M Gly solution. The concentration of Gly monomers remaining on the montmorillonite gradually decreased with increasing heating time (Fig. 3-1). As shown in Table 3-1, after heating for 336 h, 45.6 % of the Gly remained adsorbed on montmorillonite under dry conditions, while in the wet conditions, 73.3 % of the Gly was still adsorbed on the montmorillonite.

As a reference, Gly was heated without montmorillonite, and 99.1 % and 93.8 % of Gly remained under dry and wet conditions, respectively.

3.1.2 Gly peptide formation

Figure 3-2 and Table 3-2 shows the changes in the yield of Gly peptides (ratio of produced peptide/initial Gly in %) as a function of heating time, and Table 1 shows the yield after heating for 336 h. Three types of peptides (DKP, Gly_2, and Gly_3) were identified to have formed when reacted with montmorillonite under dry conditions (Fig. 3-2a), while the peptides were not detected in the wet condition.

The amount of Gly peptides on montmorillonite gradually increased with heating time, and a total of 13.9 % of the primary Gly formed peptides in the dry condition after 336 h. DKP was consistently the main product and the yields of Gly_2 and Gly_3 were low.

When Gly was heated without montmorillonite, 0.7 % of the Gly formed peptides in the wet condition after 336 h (Fig. 3-2b), while no peptides were found under the dry

conditions. Although a small amount of DKP and Gly_2 were synthesized, Gly_3 was not detected when only Gly was heated with water.

3.1.3 Dry–Wet Experiment

49.7% of the Gly monomer remained after the dry–wet conditions experiment with montmorillonite was finished (Table 3-1), and 0.7% of it transformed into peptides (Table 3-2). Most of the DKP was decomposed after the system was heated again with additional water for 168 h, and Gly_2 became the most abundant peptide under these conditions.

3.1.4 XRD analysis

Figure 3-3 shows the X-ray diffractograms of Na- and Gly-montmorillonite. The basal spacing of Na-montmorillonite (001) was 12.8 Å (Fig. 3-3a). After shaking in solutions of 0.1 M and 1 M Gly and freeze-drying, the basal spacing of Gly-montmorillonite increased to 13.2 (Fig. 3-3b) and 16.1 Å (Fig. 3-3c), respectively (Table 3-2).

3.1.5 FT-IR analysis

Figure 3-4 shows the FT-IR spectra of the studied montmorillonites. The band at 1035 cm^{-1} corresponds to the Si−O−Si stretching vibration. The band at 3618 cm^{-1} indicates OH stretching, and the bands at 3421 and 1627 cm^{-1} are from the stretching and bending vibrations of adsorbed water (Naidja and Huang, 1994; Cuadros et al., 2009). Gly-montmorillonite has bands between 1000 and 2000 cm^{-1} from the asymmetric deformation of NH_3^+ (δ_{as}) (1593cm^{-1}), the symmetric deformation of NH_3^+ (δ_s) (1506 cm^{-1}), the symmetric stretching of COO^- (ν_s) (1404 cm^{-1}) and the rocking of CH_2 (ρ_r)

(1339 cm^{-1}) (Fig. 3-4a) (Sato, 1999; Kollar et al., 2003). Figure 3-5 shows an enlargement of the spectra between 1200 and 2000 cm^{-1}. The Gly-montmorillonite has bands at 1634, 1523, 1412 and 1331 cm^{-1}, respectively (Fig. 3-5c and Table 3-4). The band of NH_3^+ (δ_{as}) at 1634 cm^{-1} overlaps with the band of OH at 1627cm^{-1}. The peak area of the band at 1627cm^{-1} is 306.4, but it considerably increases to 2075.8 after Gly adsorption. After heating for 288 h under the dry conditions, bands are observed at 1637, 1522, 1419, and 1334 cm^{-1} (Fig. 3-5d). An additional band appears at 1671 cm^{-1}, which corresponds to the carbonyl band (−C(=O)−) of DKP (Lin et al., 2002). When reacted under wet conditions, the product has bands of NH_3^+ (δ_{as}) at 1637 cm^{-1}, NH_3^+ (δ_s) at 1522 cm^{-1}, COO^- (ν_s) at 1419 cm^{-1} and CH_2 (ρ_r) at 1338 cm^{-1} (Fig. 3-5e).

3.2 Olivine experiment

3.2.1 Formation of Ala-DKP

Figure 3-6 and Table 3-5 show the changes in the yields of Ala-DKP (produced DKP/initial Ala ratio in %) in the systems after heating for 8 days. When the DL-Ala was heated without olivine, 3.0 % DL-Ala transformed into Ala-DKP. On the other hand, 11.7 % of DL-Ala changed to Ala-DKP when the olivine coexisted. The DKP were not detected even if the olivine coexisted after heating for 8 days, when a small amount of water was not added.

3.2.2 Diastereoisomers of Ala-DKP

The yields of *cis*-DKP and *trans*-DKP after heating for 8 days were close to the same amount; 1.6 % and 1.4 %, respectively (Fig. 3-7 and Table 3-5).When DL-Ala was heated with olivine, the amount of *cis*-DKP and *trans*-DKP after heating for 8 days were

6.8 % and 4.9 %, respectively, while the amount of *trans*-DKP (2.0 %) was temporarily higher than that of *cis*-DKP (1.8 %) after for 2 days.

3.3 Hydrothermal Experiment

3.3.1 DFAA in hydrothermal solutions

Figure 3-8 and Table 3-6a show the changes in the concentrations of DFAA dissolved in the hydrothermal solutions at 150 °C as a function of heating time. Although the concentration of DFAA was increased to 111.1 μM after heating for 48 h, it was decreased to 20.5 μM after 240 h.

The compositions of DFAA (Mol %) in the solution after heating at 150 °C for 24−240 h are shown in Fig. 3-9 and Table 3-6b. Gly (21 %) was the most abundant (61.2 %), followed by Ala (10.6 %) and Asp (6.2 %) after heating for 24 h. The molar percentage of Gly was gradually decreased and 17.9 % after 240 h. On the other hand, Lys, which was only 3.8 % after 24 h, was increased to 29.4 % after 240 h. Glu, Val, Ile, Leu, and Phe were also slightly increased after 240 h. Met, Tyr, and Arg were not detected in the solution after 240 h.

3.3.2 DCAA in hydrothermal solutions

The concentration of DCAA in the studied hydrothermal solutions was calculated by subtracting DFAA from THAA:

$$\text{DCAA} = \text{THAA} - \text{DFAA}$$

Figure 3-8 and Table 3-7a show the changes in the concentrations of DCAA dissolved

in the hydrothermal solutions at 150 °C as a function of heating time. DCAA was rapidly released into the solution after starting the reaction and gradually decreased with increasing heating time. The concentrations of DCAA after heating for 24 h and 240 h were 193.3 μM and 98.2 μM, respectively. The concentration of DCAA was consistently higher than that of DFAA in the solution.

The compositions of DFAA (Mol %) in the solution after heating at 150 °C for 24−240 h are shown in Fig. 3-10 and Table 3-7b. Most of the DCAA consist of Gly (25.8 %), Glu (17.3 %), Ala (13.6 %), Val (10.6 %), and Asp (9.8 %) after heating for 24h. The molar percentages of Gly and Asp were remarkably decreased to 11.2 % and 2.0 % after 240 h, respectively. Thr, Ser, Ala, Val, Lys, and His were slightly decreased after 240 h. On the other hand, Glu was increased to 50.6 % after 240 h.

3.3.3 DFAA and DCAA in the solution at different temperature conditions

Figure 3-11 and Table 3-8 show the concentrations of DFAA and DCAA in the hydrothermal solutions after heating at 100, 150, and 200 °C for 240 h. The concentrations of DFAA in the solution were 30.9, 20.5, and 7.0 μM at 100, 150, and 200 °C, respectively. The concentrations of DCAA in the solution were 311.1, 98.2, and 76.0 μM at 100, 150, and 200 °C, respectively, and it was higher than that of DFAA.

Figure 3-12 and Table 3-9 show the changes in compositions of DFAA and DCAA (Mol %) in the solution after heating at the different temperature for 240 h. In DFAA, the molar percentages of Asp, Ser, Gly, Ala, and Ile were decreased and Glu, Phe, and Lys were increased with increasing temperature. In DCAA, Asp (15.6 %), Glu (11.2 %), Gly (21.1 %), Ala (11.0 %), and Val (10.8 %) were abundant in the solution heated at 100 °C. However, most of DCAA composed by Glu (88.6 %) in the solution at 200 °C

3.3.4 THAA in the sediments

Initial concentration of THAAs in the seafloor sediment was 14312.5 nmol/g, and Gly (21 %) and Asp (18 %) were abundant, followed by Ala (11 %), Val (9 %), Thr (8 %) and Ser (8 %) (Table 3-10a). Met and Tyr were not detected. After heating for 240 h, the concentration was decreased to 1017.9 nmol/g, and Glu (17.4 %), Asp (15.9), and Gly (13.0 %) were abundant (Table 3-10b).

Table 3-1 Amount of Gly remaining (%) after heating at 150 °C for 24–336 h

Samples	Condition	Remaining Gly (%)					
		24 h	48 h	96 h	168 h	240 h	336 h
Montmorillonite	Dry	69.2	54.6	45.9	43.9	46.3	45.6
Montmorillonite	Wet	n.d.	76.7	71.9	72.2	71.7	73.3
Montmorillonite	Dry-Wet	n.d.	n.d.	n.d.	n.d.	n.d.	49.7
Reference	Dry	n.d.	98.2	97.6	98.1	99.0	99.1
Reference	Wet	n.d.	98.8	99.1	95.9	95.7	93.8

Table 3-2 Amount of Gly peptide yield (%) after heating at 150 °C for 24–336 h

Samples		Yield (%)				
		48h	96h	168h	240h	336h
Montmorillonite (Dry)	DKP	4.2	5.3	7.5	10.6	12.0
	Gly_2	0.7	0.8	1.1	1.6	1.6
	Gly_3	0.2	0.2	0.3	0.3	0.3
	Total	5.1	6.3	8.8	12.5	13.9
Montmorillonite (Dry-Wet)	DKP	n.d	n.d	n.d	n.d	0.4
	Gly_2	n.d	n.d	n.d	n.d	0.3
	Total	n.d	n.d	n.d	n.d	0.7
Reference (Wet)	DKP	0.1	0.2	0.3	0.4	0.4
	Gly_2	0.1	0.1	0.2	0.2	0.3
	Total	0.2	0.4	0.5	0.6	0.7

Table 3-3 Basal spacing of montmorillonite before and after shaking with 100 mM or 1000 mM Gly solutions

Samples	Gly solution concentration (mM)	2θ (degree)	d_{001} Å
(a) Na-montmorillonite	0	6.9	12.8
(b) Gly-montmorillonite	100	6.7	13.2
(c) Gly-montmorillonite	1000	5.5	16.1

Table 3-4 Wavenumber (cm^{-1}) of NH_3^+ (δ_{as}), NH_3^+ (δ_s), COO^- (v_s) and CH_2 (ρ_r) derived from Gly

Samples	Wavenumber (cm^{-1})			
	CH_2 (ρ_r)	COO^- (v_s)	NH_3^+ (δ_s)	NH_3^+ (δ_{as})
(a) Gly (pure)	1339	1404	1506	1593
(c) Gly-montmorillonite	1331	1412	1523	1634
(d) Gly-montmorillonite after heating (dry)	1334	1522	1419	1637
(e) Gly-montmorillonite after heating (wet)	1338	1419	1552	1637

Table 3-5 Amount of Ala-DKP yield (%) after heating at 120 °C for 8 days

	Yield (%)							
Heating time (day)	1	2	3	4	5	6	7	8
with olivine								
cis DKP	1.1	1.8	2.8	3.3	4.2	4.9	6.0	6.8
trans DKP	1.2	2.0	2.5	2.9	3.7	4.1	4.6	4.9
Total	**2.3**	**3.7**	**5.3**	**6.2**	**7.9**	**9.0**	**10.6**	**11.7**
without olivine								
cis DKP	0.1	0.2	0.5	0.8	1.0	1.2	1.6	1.6
trans DKP	0.1	0.2	0.4	0.7	0.9	1.2	1.4	1.4
Total	**0.2**	**0.3**	**0.9**	**1.6**	**1.9**	**2.4**	**3.0**	**3.0**

Table 3-6 changes in the (a) concentrations (μM) and (b) compositions of DFAA (Mol %) in the hydrothermal solutions at 150 °C for 240 h

(a)

Heating time (h)	DFAA (μM)															
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Total
24	5.4	0.3	2.8	1.0	52.9	9.2	3.5	0.0	1.8	2.4	0.0	3.3	3.3	0.1	0.3	86.5
48	7.9	0.9	2.4	1.2	66.5	14.0	5.4	0.0	2.0	2.8	0.0	3.4	4.1	0.1	0.4	111.1
96	4.0	0.4	1.1	1.3	54.7	15.8	6.6	0.0	1.8	2.7	0.0	3.3	5.2	0.1	0.0	97.0
144	2.3	0.1	1.0	1.1	25.8	11.2	6.2	0.0	1.6	2.3	0.0	2.9	6.4	0.1	0.0	61.2
192	1.8	0.1	1.0	1.0	15.2	8.6	5.8	0.0	1.7	2.1	0.0	2.7	6.0	0.2	0.0	46.2
240	0.9	0.1	0.8	0.7	3.7	2.2	2.8	0.0	0.8	0.9	0.0	1.6	6.0	0.1	0.0	20.5

(b)

Heating time (h)	DFAA (Mol %)														
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg
24	6.2	0.4	3.2	1.2	61.2	10.6	4.1	0.0	2.1	2.8	0.0	3.9	3.8	0.1	0.4
48	7.1	0.8	2.2	1.1	59.8	12.6	4.8	0.0	1.8	2.5	0.0	3.0	3.7	0.1	0.4
96	4.1	0.4	1.1	1.3	56.4	16.3	6.8	0.0	1.9	2.8	0.0	3.4	5.4	0.1	0.0
144	3.8	0.2	1.7	1.8	42.1	18.3	10.2	0.0	2.7	3.8	0.0	4.7	10.5	0.1	0.1
192	3.9	0.3	2.1	2.1	32.9	18.7	12.5	0.0	3.7	4.4	0.0	5.9	13.1	0.5	0.0
240	4.3	0.6	3.7	3.4	17.9	10.8	13.8	0.0	3.8	4.3	0.0	7.7	29.4	0.4	0.0

Table 3-7 changes in the (a) concentrations (μM) and (b) compositions of DCAA (Mol %) in the hydrothermal solutions at 150 °C for 240 h

(a)

Heating time (h)	DCAA (μM)															
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Total
24	18.9	8.6	4.0	33.4	50.0	26.2	20.6	0.0	5.9	8.4	0.0	6.1	6.2	1.2	3.8	193.3
48	12.8	4.2	1.4	39.5	41.6	23.6	20.5	0.0	5.6	8.9	0.0	7.3	6.3	1.0	3.0	175.5
96	10.5	2.1	2.6	46.6	28.7	18.9	16.7	1.0	5.0	9.2	0.0	6.3	4.3	0.9	1.8	154.5
144	6.6	0.6	0.2	49.1	18.4	14.2	13.1	0.2	4.3	7.2	0.1	5.4	3.1	0.3	0.6	123.5
192	5.6	0.6	1.3	50.2	15.9	12.3	12.2	0.6	4.1	7.9	0.0	6.0	2.9	0.7	0.2	119.9
240	2.0	0.5	1.3	49.7	11.0	8.8	9.6	0.7	3.2	5.5	0.0	4.9	1.0	0.2	0.0	98.2

(b)

Heating time (h)	DCAA (Mol %)														
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg
24	9.8	4.4	2.1	17.3	25.8	13.6	10.6	0.0	3.0	4.4	0.0	3.2	3.2	0.6	2.0
48	7.3	2.4	0.8	22.5	23.7	13.4	11.7	0.0	3.2	5.0	0.0	4.1	3.6	0.6	1.7
96	6.8	1.4	1.7	30.2	18.5	12.2	10.8	0.7	3.2	5.9	0.0	4.1	2.8	0.6	1.2
144	5.4	0.5	0.1	39.8	14.9	11.5	10.6	0.2	3.5	5.9	0.1	4.4	2.5	0.3	0.5
192	4.6	0.5	1.1	41.9	13.2	10.3	10.2	0.5	3.4	6.6	0.0	5.0	2.4	0.6	0.2
240	2.0	0.5	1.3	50.6	11.2	8.9	9.7	0.8	3.2	5.6	0.0	5.0	1.1	0.2	0.0

Table 3-8 Concentrations (μM) of (a) DCAA and (b) DFAA in the hydrothermal solutions at 100, 150 and 200 °C for 240 h

(a)

Temperature (°C)	DFAA (μM)															
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Total
100	4.5	0.9	2.7	1.2	11.9	3.8	1.1	0.0	0.6	0.7	0.0	2.0	1.2	0.1	0.1	30.9
150	0.9	0.1	0.8	0.7	3.7	2.2	2.8	0.0	0.8	0.9	0.0	1.6	6.0	0.1	0.0	20.5
200	0.0	0.0	0.2	0.7	1.1	0.1	0.2	0.0	0.1	0.3	0.0	0.7	3.5	0.1	0.0	7.0

(b)

Temperature (°C)	DCAA (μM)															
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Total
100	48.4	19.3	15.9	34.8	65.7	34.4	33.7	0.0	11.3	15.4	0.0	11.8	10.0	3.3	7.2	311.1
150	2.0	0.5	1.3	49.7	11.0	8.8	9.6	0.7	3.2	5.5	0.0	4.9	1.0	0.2	0.0	98.2
200	0.2	0.1	0.0	67.4	1.2	0.2	0.9	0.0	0.9	1.1	0.0	1.2	0.5	2.4	0.1	76.0

Table 3-9 Compositions (Mol %) of (a) DCAA and (b) DFAA in the hydrothermal solutions at 100, 150 and 200 °C for 240 h

(a)

Temperature (°C)	DFAA (Mol %)														
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg
100	14.6	2.9	8.9	4.0	38.4	12.3	3.6	0.0	2.1	2.3	0.0	6.6	3.8	0.3	0.4
150	4.3	0.6	3.7	3.4	17.9	10.8	13.8	0.0	3.8	4.3	0.0	7.7	29.4	0.4	0.0
200	0.0	0.2	2.9	10.4	15.5	1.6	2.7	0.0	1.0	4.3	0.0	10.1	50.2	1.2	0.0

(b)

Temperature (°C)	DCAA (Mol %)														
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg
100	15.6	6.2	5.1	11.2	21.1	11.0	10.8	0.0	3.6	4.9	0.0	3.8	3.2	1.0	2.3
150	2.0	0.5	1.3	50.6	11.2	8.9	9.7	0.8	3.2	5.6	0.0	5.0	1.1	0.2	0.0
200	0.2	0.1	0.1	88.6	1.5	0.2	1.2	0.0	1.2	1.5	0.0	1.5	0.7	3.2	0.1

Table 3-10 Concentrations and compositions of THAA in sediments before and after heating at 150 °C for 240 h

(a)

	THAA (nmol/g)															
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Total
Before heating	2306.7	1027.6	1029.9	1298.3	2749.6	1482.8	1236.3	0.0	435.5	615.6	0.0	473.7	867.2	180.9	608.4	14312.5
After heating for 240 h	161.8	16.8	28.9	176.6	132.6	93.7	95.7	0.0	56.5	74.0	0.0	90.0	67.6	12.4	11.4	1017.9

(b)

	THAA (%)														
	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg
Before heating	16.1	7.2	7.2	9.1	19.2	10.4	8.6	0.0	3.0	4.3	0.0	3.3	6.1	1.3	4.3
After heating for 240 h	15.9	1.6	2.8	17.4	13.0	9.2	9.5	0.0	5.5	7.2	0.0	8.9	6.7	1.2	1.1

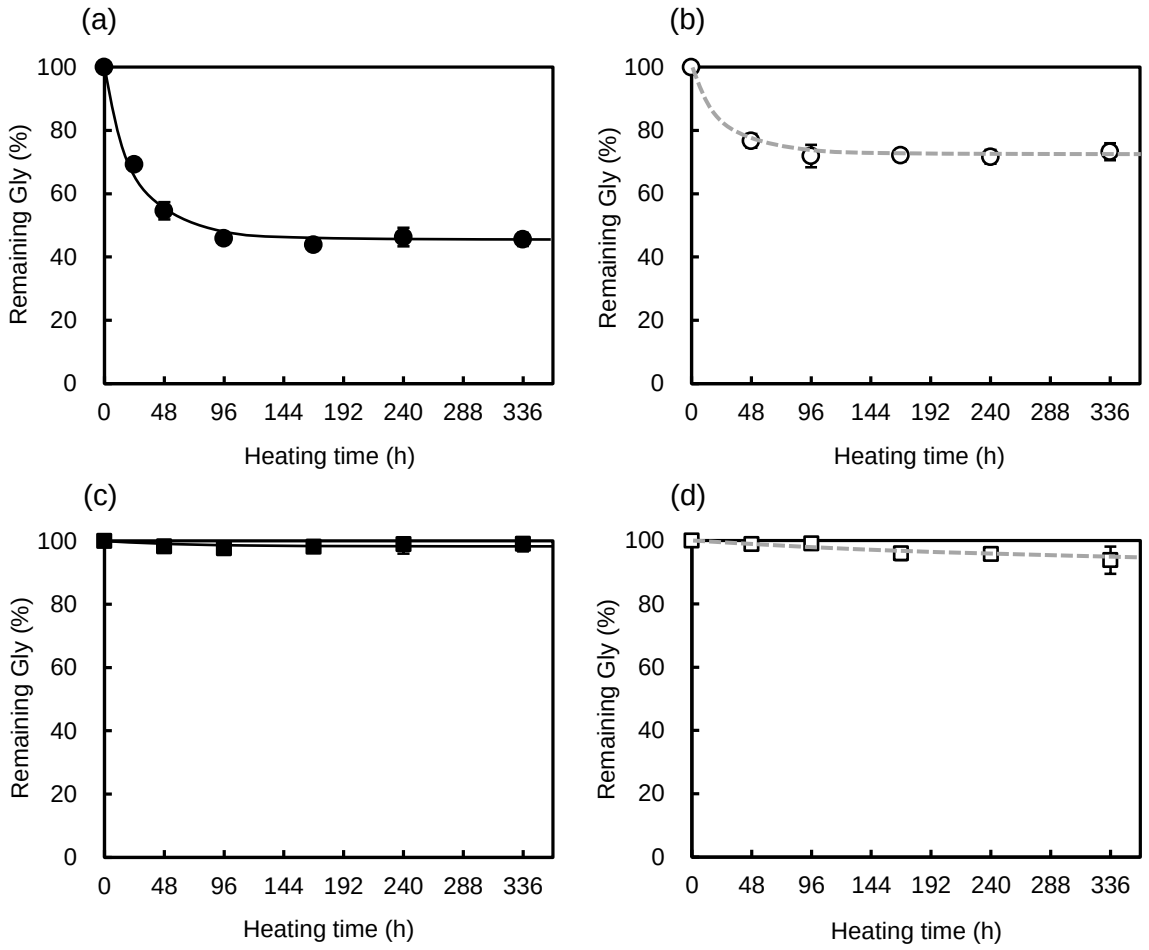


Fig. 3-1 Changes in the remaining Gly (%) after heating at 150 °C for 336 h (a) with montmorillonite under dry conditions, (b) with montmorillonite under wet conditions, (c) without montmorillonite under dry conditions and (d) without montmorillonite under wet conditions

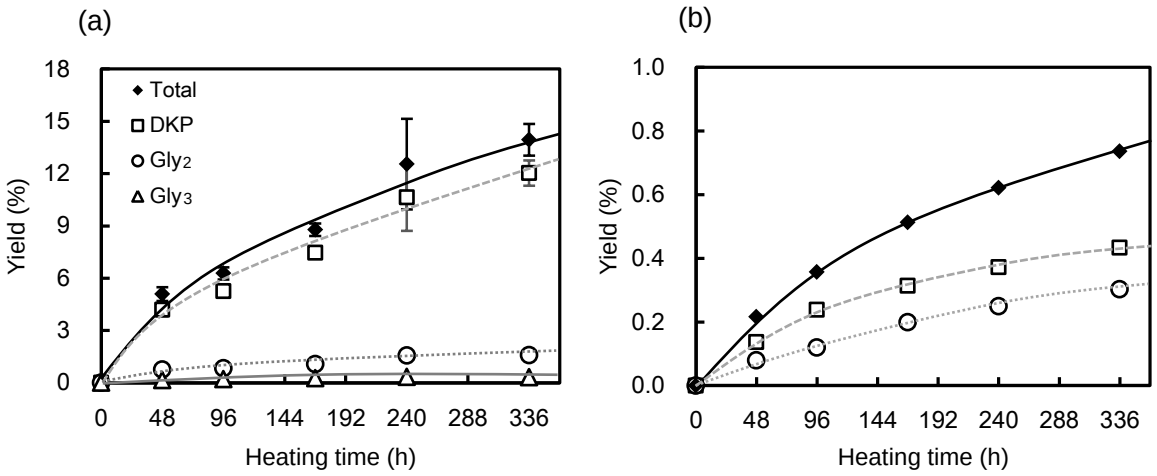


Fig. 3-2 Yield of peptides (a) on montmorillonite under dry conditions and (b) without montmorillonite under wet conditions.

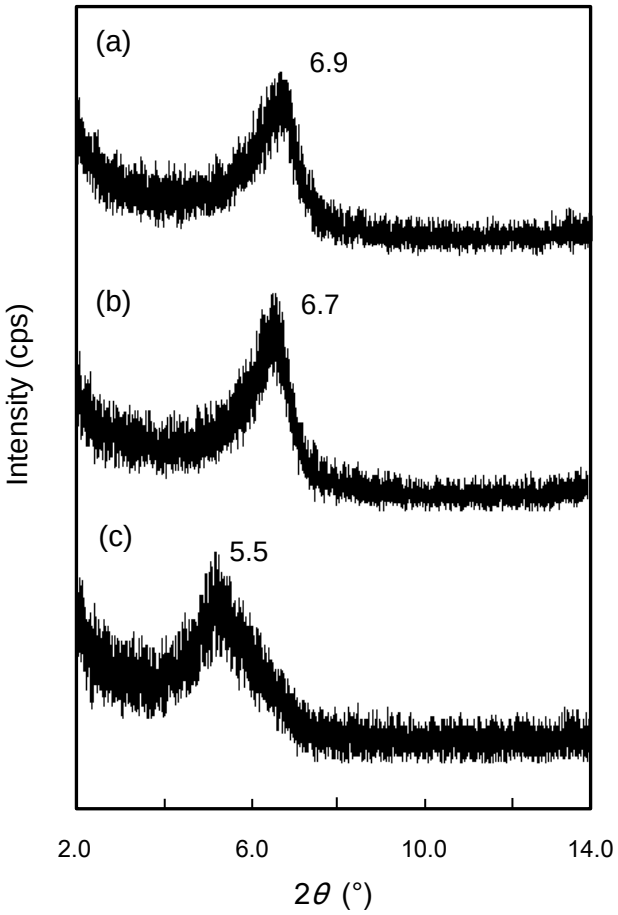


Fig. 3-3 X-ray diffractograms for 2θ angle between 2° and 14°. (a) Na-montmorillonite, (b) Gly-montmorillonite (100mM), and (c) Gly-montmorillonite (1000 mM)

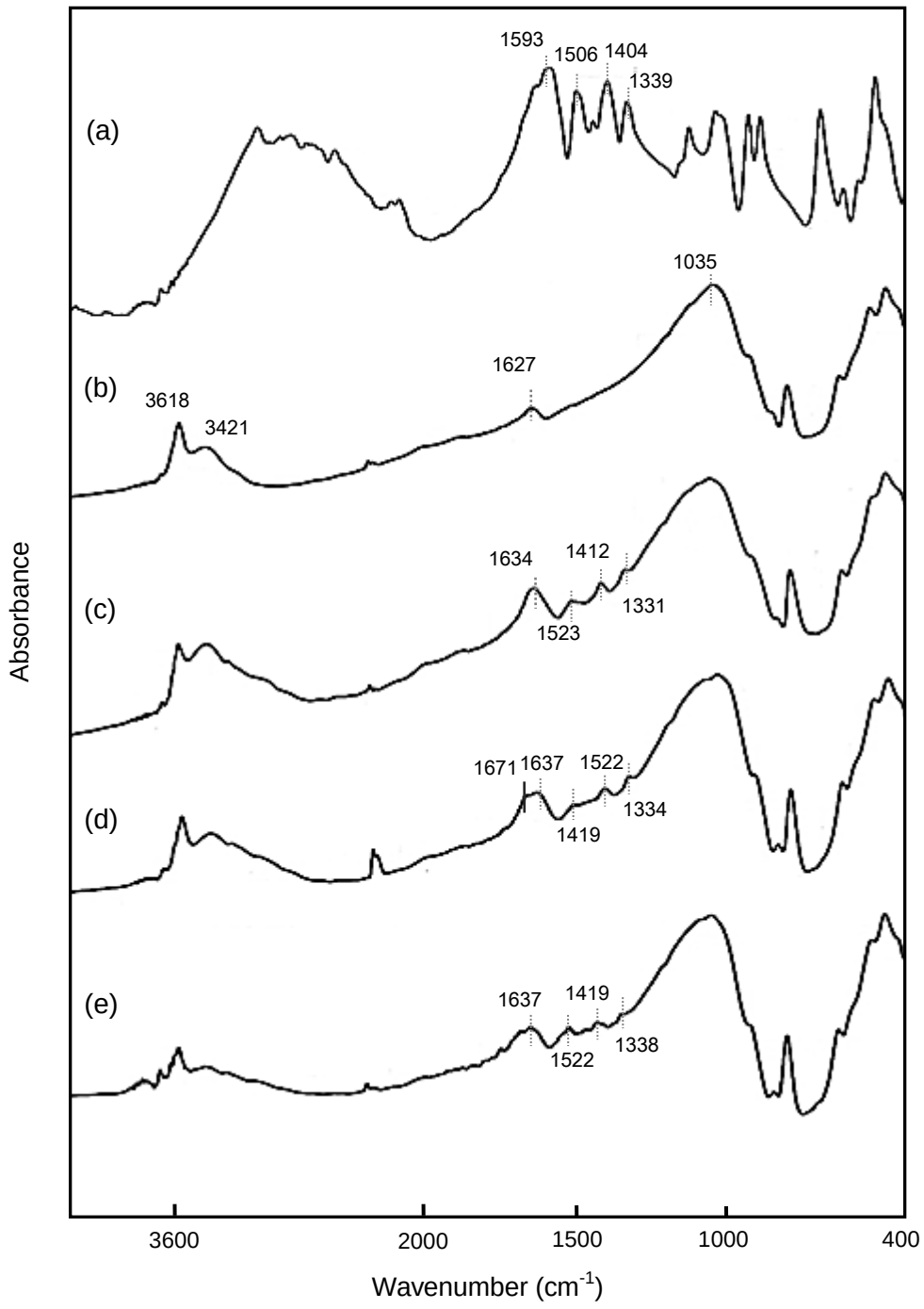


Fig. 3-4 FT-IR spectra between 400 and 4000 cm^{-1}. (a) Gly monomer, (b) Na-montmorillonite, (c) Gly-montmorillonite, (d) Gly-montmorillonite after heating at 150 °C under dry conditions and (e) Gly-montmorillonite after heating at 150 °C under wet conditions for 228 h. In (a), the band at 1593 cm^{-1} represent asymmetric deformation of NH_3^+ (δ_{as}), the band at 1506 cm^{-1} is symmetric deformation of NH_3^+ (δ_s), the band at 1404 cm^{-1} is symmetric stretching of COO^- (v_s) and the band at 1339 cm^{-1} is rocking of CH_2 (ρ_r) of pure Gly

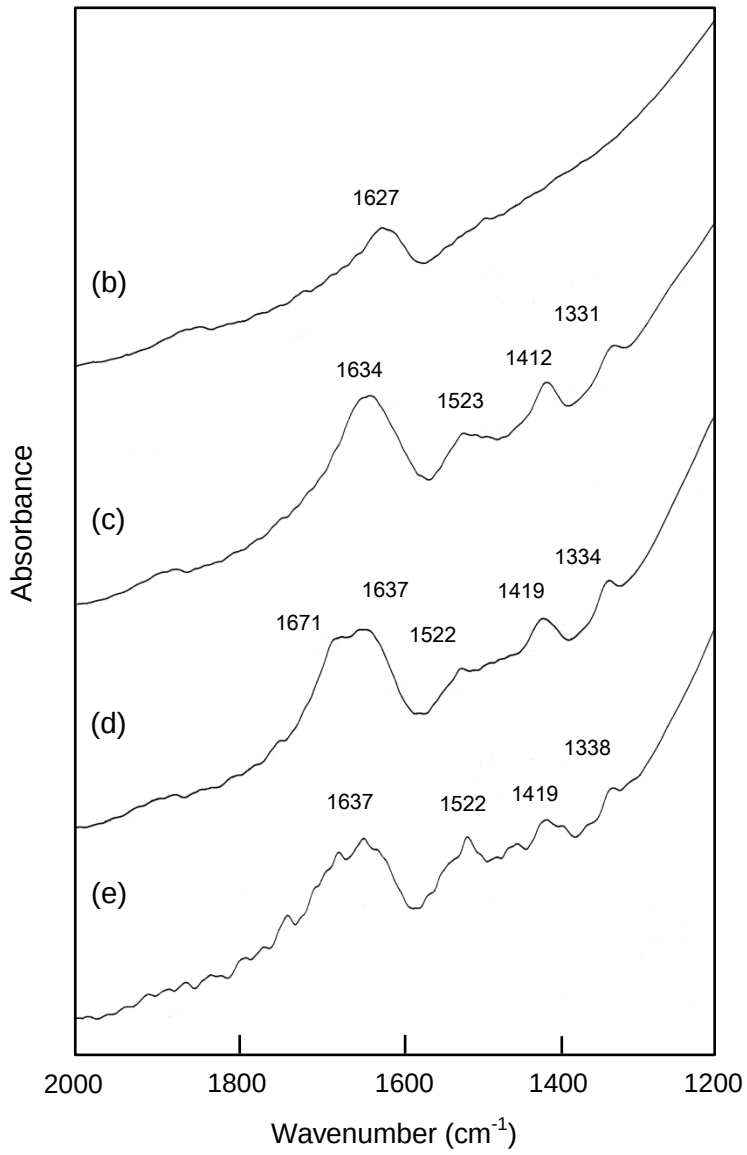


Fig. 3-5 The spectra (b), (c), (d) and (e) extended between 1200 and 2000 cm^{-1}

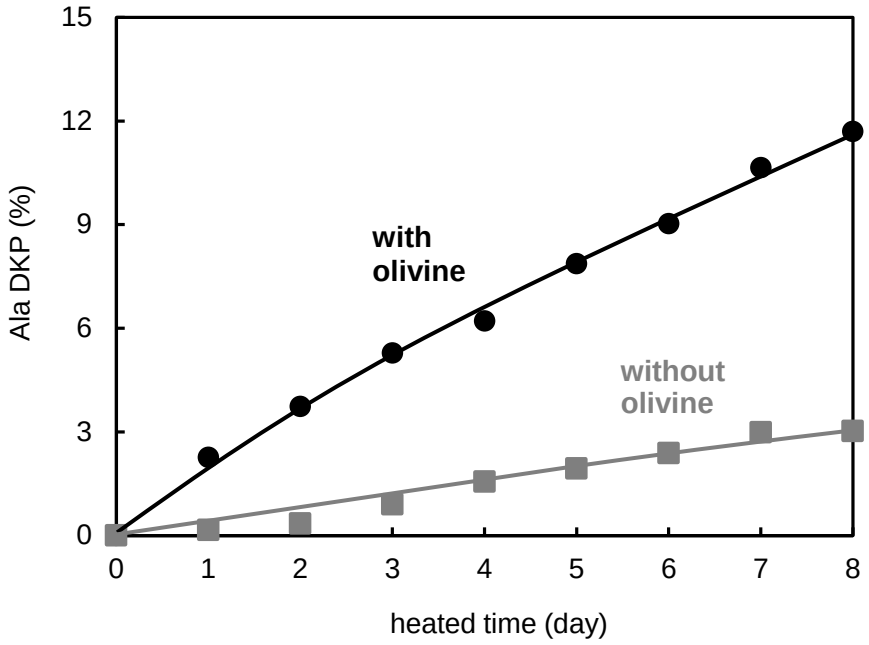


Fig. 3-6 Changes in the yield of Ala-DKP after heating for 8 days

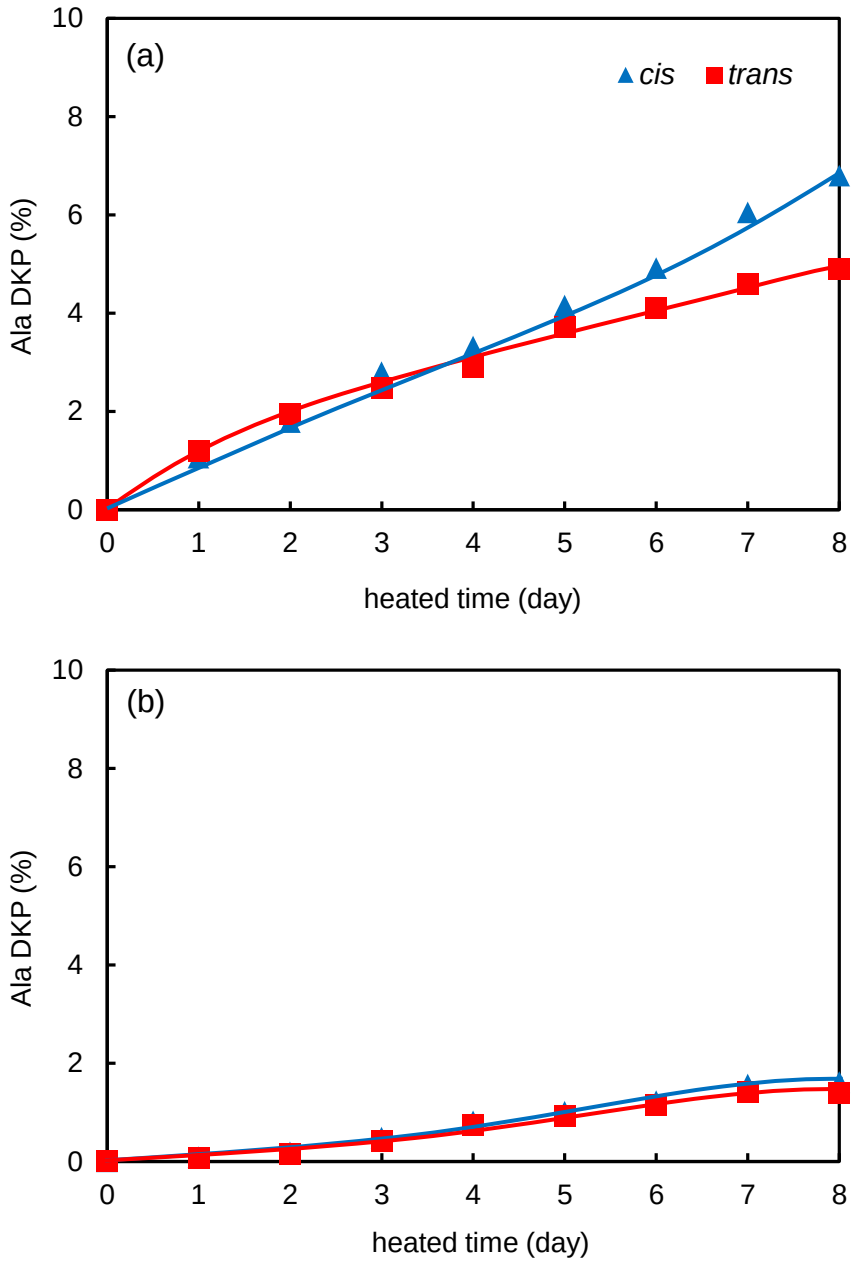


Fig. 3-7 Changes in the yield of diastereoisomers of DKP (a) with olivine (b) without olivine after heating for 8 days

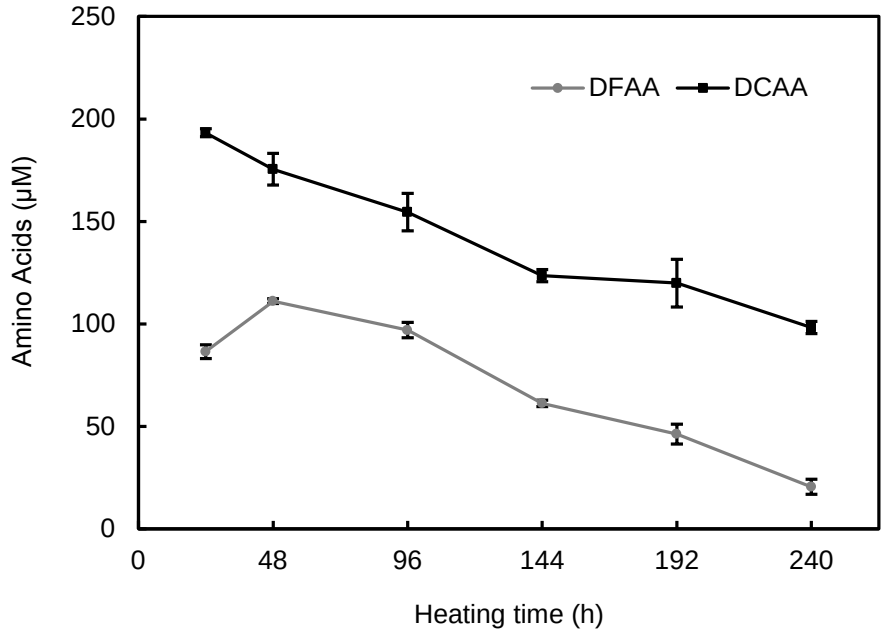


Fig. 3-8 Changes in the concentrations of DCAA and DFAA (μM) in the hydrothermal solutions after heating at 150 °C for 240 h

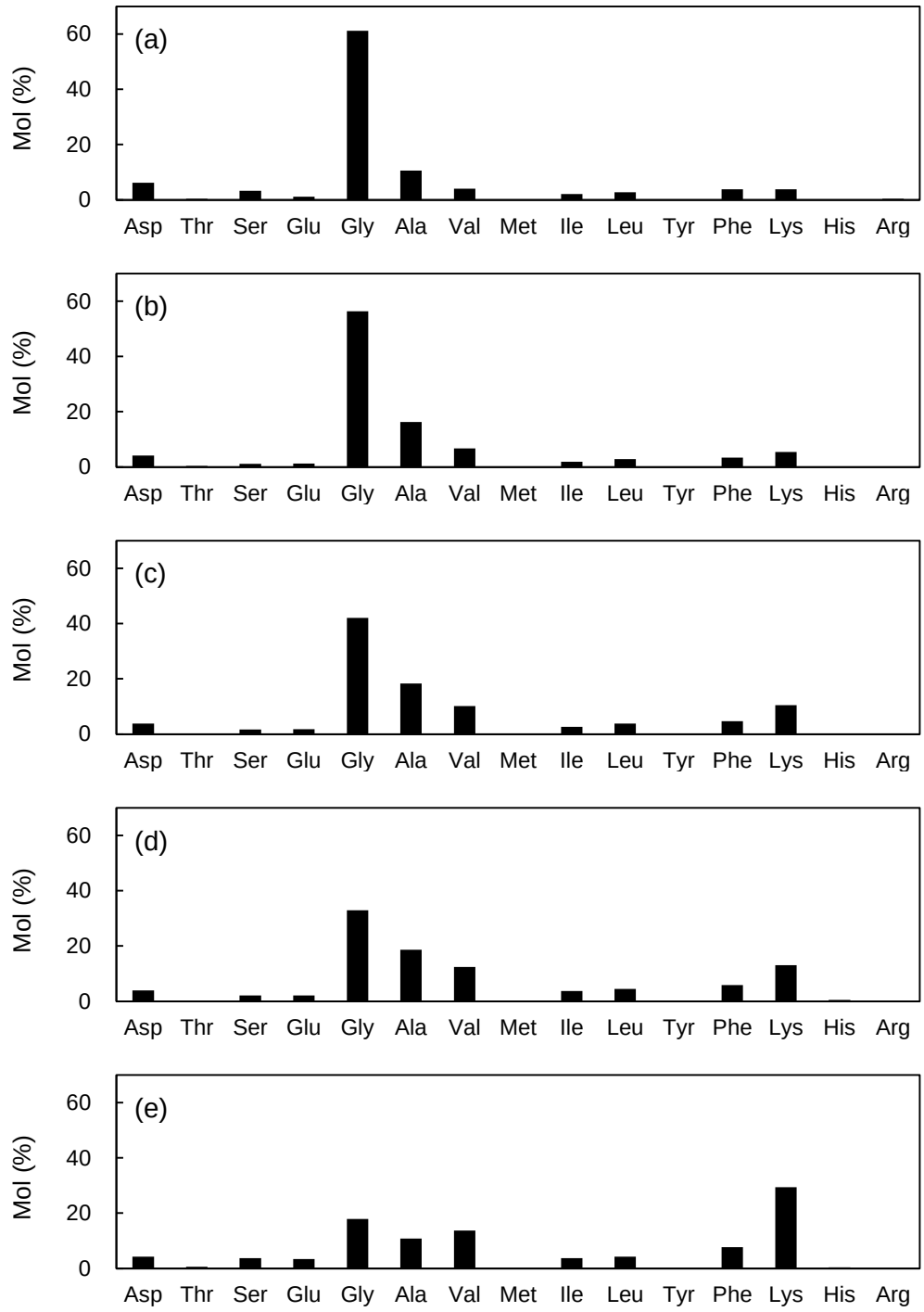


Fig. 3-9 Compositions of DFAA (Mol %) in the hydrothermal solutions after heating at 150 °C for (a) 24 h, (b) 96 h, (c) 144 h, (d) 192 h, and (e) 240 h

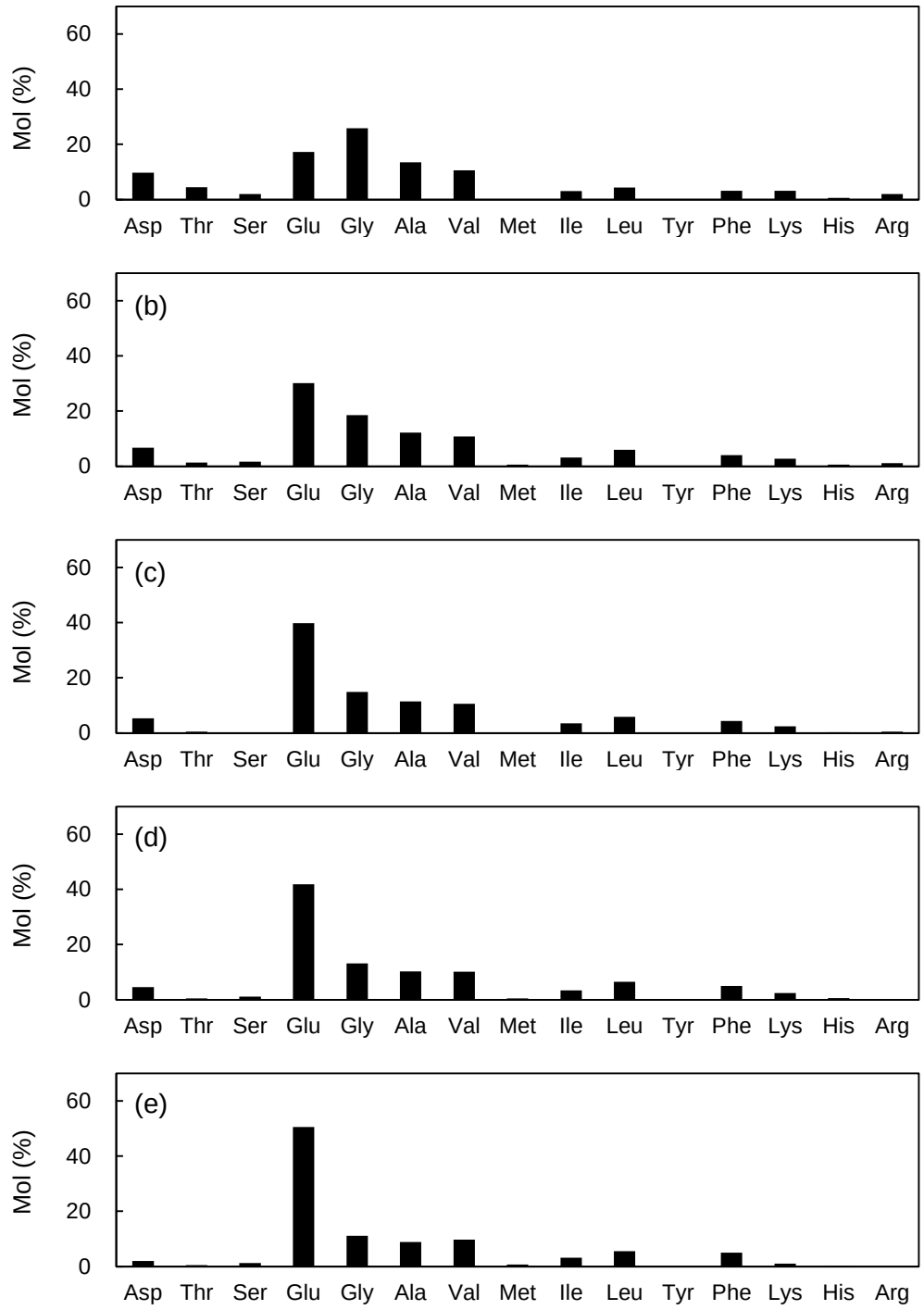


Fig. 3-10 Compositions of DCAA (Mol %) in the hydrothermal solutions after heating at 150 °C for (a) 24 h, (b) 96 h, (c) 144 h, (d) 192 h, and (e) 240 h

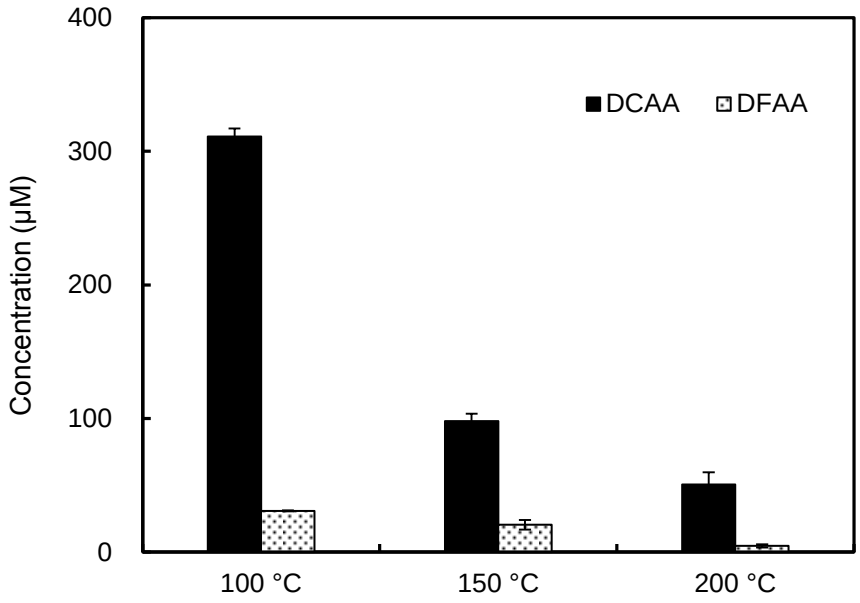


Fig. 3-11 Concentrations of DFAA and DCAA (μM) in the hydrothermal solutions after heating at 100, 150, and 200 °C for 240 h

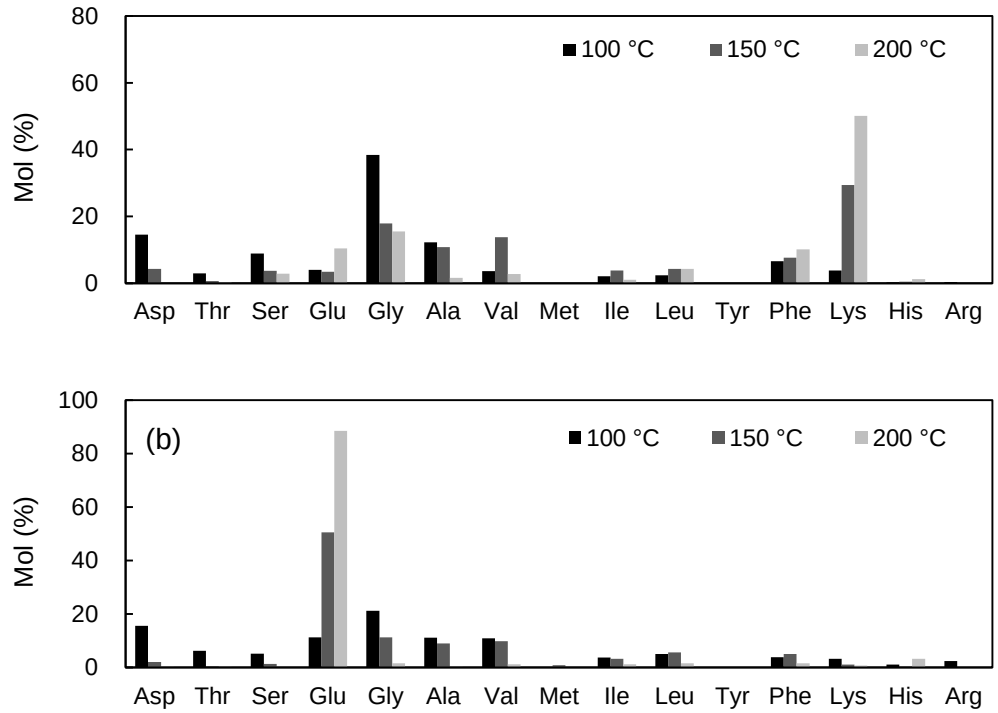


Fig. 3-12 Changes in the compositions (%) of (a) DFAA and (b) DCAA in the hydrothermal solutions after heating at 100, 150 and 200 °C for 240 h

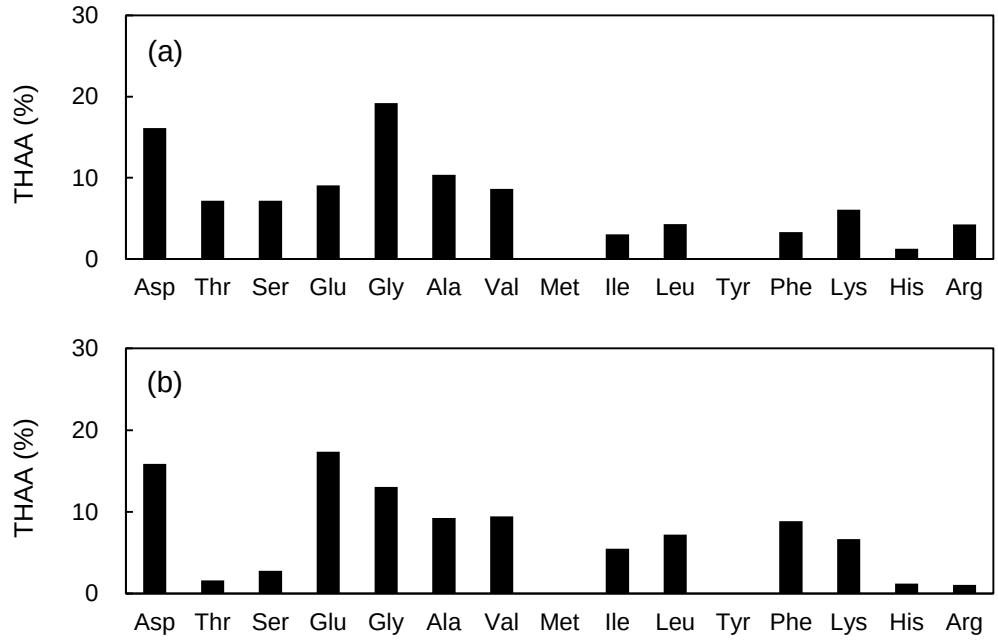


Fig. 3-13 Compositions (%) of THAA in the sediment (a) before and (b) after heating at 150 °C for 240 h

4. Discussion

4.1 Peptide formation of Gly on montmorillonite

4.1.1 Adsorption state of Gly on montmorillonite

Amino acids are commonly adsorbed onto the interlayer of smectite group minerals (Greenland et al., 1964). The basal spacing (001) expands when amino acids occupy the interlayer space of montmorillonite (Fig. 3-3 and Table 3-3) (Barshad, 1952; Naidja and Huang, 1994). Based on the CEC of the montmorillonite used, 855 μmol/g Gly could be substituted into the exchangeable site. The amount of Gly adsorbed on the montmorillonite was 234.7 μmol/g and 784.0 μmol/g when the montmorillonite was treated with 0.1 M and 1 M Gly solutions, respectively. The interlayer spacing of the Na-montmorillonite interlayer increased from 12.8 Å to 13.2 and 16.1 Å after shaking in solutions of 0.1 M and 1 M Gly respectively, confirming that Gly moved from solution into the interlayer. Gly molecules weakly adsorbed on the surface and edges of montmorillonite, and then might have desorbed when the montmorillonite was washed twice with ultra-pure water because the activation energy for desorption is small (Lambert, 2008). Amino acids are more strongly adsorbed in the interlayer than on the surface (Friebele et al., 1980). Under the conditions of this study, most of the Gly is expected to remain as interlayer cations.

It was estimated that cation exchange is a main adsorption mechanism of amino acids to the interlayer of smectite minerals (Greenland et al., 1964; Naidja and Huang, 1994). Friebele et al., (1980) observed changes in the amount of Gly adsorbed on Na-montmorillonite at pH 3–10, and found that the amount of adsorbed Gly is markedly high at pH 3. The ionic state of amino acids depends on pH condition of the solution; it

exists as cation, zwitterion and anion under acidic, neutral and basic conditions, respectively (Lambert, 2008). The changes in the ionic state of Gly as a function of pH were shown in Fig. 4-1 (Sakata et al., 2010). At pH 3, Gly exists as cation, estimating that cation exchange would be the main mechanism for amino acid adsorption to the interlayer.

However, the majority of Gly in the studied solution existed as zwitterions rather than cations, since the pH of the Gly solution was 6.4. Parbhakar et al. (2007) suggested that proton transfer was an important mechanism for amino acid zwitterions to occupy the interlayers. The reaction is described as

$$AA^{\pm} + (Na^{+}, H_2O)_{clay} \rightarrow (AA^{+}, Na^{+}, OH^{-})_{clay} \rightarrow (AA^{+})_{clay} + Na^{+} + OH^{-}$$

The amino acid zwitterions would change to cation in the interlayer via proton transfer. The FT-IR spectra indicated that the interlayer Gly was in a cationic state. The ratio of the peak areas of δ_{as} NH_3^+ before and after adsorption on montmorillonite did not greatly decrease (0.85), while that for ν_s COO^- considerably decreased (0.49). In addition, proton transfer in the interlayer space must have increased the pH of the solution after shaking owing to dissociation of H_2O and dissolution of OH^-. In fact, the pH value of the Gly solution increased from 6.3 to 7.3 (±0.1) after shaking with Na-montmorillonite. Thus, proton transfer would have been important to substitute interlayer cations with Gly in this study.

Amino acids are likely to be adsorbed in the interlayer via hydrogen bonding interactions based on the IR bands of the amino acids (Naidja et al., 1995; Kollar et al.,1990; Stievano et al., 2007; Kitadai et al., 2009). Sato (1999) suggested that the

peaks of the amino groups shifted to higher wave numbers when Gly was adsorbed in the interlayer of clay minerals via the protonated amino group (NH_3^+). In this study, the IR bands for COO^- (ν_s) and CH_2 (ρ_r) slightly shifted, while those for NH_3^+ (δ_{as}) and NH_3^+ (δ_s) significantly shifted from 1593 to 1634 cm^{-1} and 1506 to 1523 cm^{-1}, respectively (Fig. 3-5). The band of the amino group at 1593 cm^{-1} shifted to a higher wave number, suggesting that the protonated amino group (NH_3^+) played a role in the adsorption. The protonated amino group is an excellent hydrogen bond donor (Marsh and Donohue, 1967) and forms a hydrogen bond with the structural oxygen atoms of mineral surfaces (Raussel-Colom and Salvador, 1971; Naidja and Huang, 1994). Thus, the possible adsorption mechanism of Gly in the interlayer of montmorillonite would be mainly hydrogen bond between the protonated amino group of Gly (NH_3^+) and $Si{-}O^-$ and $Al{-}O^-$ groups on the montmorillonite surface (Fig. 4-2).

4.1.2 The reactivity of Gly adsorbed on montmorillonite via hydrogen bond

The thermal behavior of amino acids adsorbed on mineral surfaces via hydrogen bond is not cleared. Kanamura and Vand (1970) suggested that the hydrogen bond may stabilize the amino acids on the mineral surface. Cuadros et al. (2009) observed hydrogen bond formation between lysine and the montmorillonite surface under hydrothermal conditions (80 °C), and estimated that the hydrogen bond became stronger and stabilized with increasing temperature. In this study, however, the reactivity of the Gly monomer adsorbed on the montmorillonite increased with increasing temperature, and the amount of Gly monomer more rapidly decreased in the system with montmorillonite than in the system without montmorillonite under both dry and wet conditions. Therefore, the hydrogen bond activated rather than stabilized Gly.

When Gly was heated without montmorillonite, 99 % of the Gly remained and peptides were not detected after 336 h, although peptide formation is thermodynamically favorable under high temperature and dry conditions. Crystalline amino acids have strong hydrogen bonds between the protonated amino groups and deprotonated carboxyl groups ($NH_3^+ \cdots COO^-$), which are very stable under high temperature and dry conditions (Rosado et al., 1997; Reva et al., 1994). Sato (1999) suggested that the reactivity of amino acids adsorbed on mineral surface was higher than that of the crystalline amino acids because the strong intermolecular hydrogen bonds in the crystalline amino acid were broken and weaker hydrogen bonds between the amino acid molecules and mineral surface were formed. Thus, the activation process of adsorbed amino acids would be a determining factor for the success of the first step of peptide formation.

4.1.3 Peptide formation mechanism on montmorillonite under thermal conditions

Bujdak and Rode (1996; 1999a) observed oligomerization of Gly on some clay minerals during a dry–wet cycle experiment (80 °C, open system). They estimated that Gly was activated and oligomerized on the clay mineral surface through the formation of an ester bond (Si–O–C(=O)–R) between the carboxyl group of the amino acid (COO^-) and the silanol group (Si–OH) on the clay mineral surface and edge. Peptide formation on mineral surfaces is often explained by the nucleophilic reaction between an amino group and a carboxyl group of amino acids (Lambert, 2008). The electron density of the carbon atom of the carboxyl group of the amino acid decreases through adsorption because of ester bond formation and nucleophilic attack at the carbon atom by an amino group of another amino acid (Bujdak and Rode, 1996; 1999a; Basiuk and

Gromovoy, 1993). However, the ester bond cannot be easily formed under 100 °C, because this temperature is thermodynamically unfavorable for the formation reaction ($\Delta°G$ = 22.5 to –29.7 kJ mol^{-1}) (Rimola et al., 2006). In fact, the formation of an ester bond is observed only when amino acids are heated with silicate minerals in the dry condition system at very high temperature (> 600 °C) (e.g., Meng et al., 2004). An IR peak at 1760 cm^{-1} provides evidence of the formation of an ester bond (Basiuk et al., 1990). However, this peak has not been detected in other experiments of the simulation of peptide formation on mineral surfaces (Lomenech et al. 2005), as well as in this study. Thus, peptide formation in this study is likely to be independent of the ester bond formation on montmorillonite.

Recently, some molecular modeling studies using *ab initio* methods, which are used to calculate and simulate molecular structures based on the first principles of quantum mechanisms, revealed that the acid–base reaction, especially at Lewis and Brønsted acid/base sites on the mineral surface, is an important catalyst for the peptide formation reaction (Yu et al., 2001; Aquino et al., 2004; Rimola et al., 2005; 2007). Clay mineral surfaces have many electrophilic defects. For example, the aluminum atom (Al^{3+}) acts as a Lewis acid or base site when the hydroxyl group of the aluminol group (Al–OH) changes to $–O^-$ or $–OH_2^+$ by dehydration or change of the pH, respectively (Ward and Brady, 1998). Aquino et al. (2004) suggested that electrophilic defect sites, such as the aluminum atom that loses its hydroxyl group (i.e., becomes a Lewis acid site) on the clay mineral surface, were more suitable for the peptide formation reaction than sites covered with a hydroxyl group, such as Si–OH or Al–OH. Rimola (2007) simulated dimerization of Gly on Lewis and Brønsted acid sites on feldspars using *ab initio* methods, and revealed that the Lewis acid sites (Al^{3+}) act as a catalyst when Gly is

adsorbed via the oxygen atom of the carbonyl group. These studies simulated amino acid adsorption via the carboxyl group on a positively charged mineral surface, i.e., Lewis and Brønsted acid sites.

However, my results, and many studies using FT-IR, revealed that hydrogen bond formation between the protonated amino group and the negatively charged mineral surface is the main adsorption mechanism of amino acids. In other words, most of the amino acids were not adsorbed on the acid site but on the base site. Aquino et al. (2004) estimated that the Lewis base sites are also effective catalysts that enhance the peptide formation, because the adsorption of amino acids by hydrogen bond on the Lewis base sites was observed during molecular modeling simulations. Recently, using attenuated total reflection FT-IR spectroscopy, Ramos and Huertas (2013) found that Gly adsorbed by the carboxyl group on montmorillonite edge sites only when the concentration of the Gly solution was very low (<0.001 M) and that hydrogen bond formation was the main adsorption mechanism of amino acids on montmorillonite. In this study, 13.9 % of Gly adsorbed on montmorillonite changed into peptides after heating for 336 h under dry condition, estimating that peptide formation reaction would be enhanced via the hydrogen bond formation on the base site. Thus, I suggest that the base site on montmorillonite is more important adsorption site for amino acids and as catalyst for peptide formation than the acid site.

4.1.4 Dehydration promotion and DKP condensation on montmorillonite under dry conditions

The reactivity of the interlayer Gly of montmorillonite was considerably enhanced at dry conditions, and 40.5 % of Gly decomposed after heated for 336 h. The

decomposition pathways of Gly are divided into deamination, decarboxylation, dehydration and hydrolysis. Of these, deamination and dehydration are the main reaction pathways that occur under high temperature and dry conditions (Li et al., 2007; Otake et al., 2011). The formation of peptides under high temperature and dry conditions in this study suggests that dehydration may be the main decomposition pathway on the montmorillonite surface.

Kitadai et al. (2011) investigated the effects of anhydrous salts, where hydration is thermodynamically favorable, on peptide formation under dry conditions at 140 °C. They reported that 6.92 % of Gly formed peptides when heated for 20 days with $MgSO_4$, but only 0.035 % formed peptides in the absence of $MgSO_4$. Montmorillonite can also promote dehydration, because polar H_2O molecules are easily adsorbed on the negatively charged surface (Hensen and Smit, 2002). Paecht-Horowiz and Lahav (1977) reported that the amount of newly formed Ala peptides was lower when Ala was reacted with Al-montmorillonite than with Na-montmorillonite because Al-montmorillonite does not have the ability to adsorb water molecules. Under high temperature and dry conditions, clay minerals most likely absorb water, similar to metal and anhydrous inorganic salts, and take water from amino acids, promoting peptide formation. In particular, the minerals from the smectite group, including montmorillonite, enhance the dehydration of amino acids because of their high absorbency.

The reaction pathway of the oligomerization of Gly is shown in Fig. 4-3. Most of the peptides formed on montmorillonite during the experiment under dry conditions were in the form of DKP (86 % of the total peptides formed after heating for 336 h). DKP is easily formed via cyclization and dehydration of Gly_2. Figure. 4-4a shows how the ratio of products (Gly_2/DKP) on montmorillonite changed with heating time. The ratio

gradually decreased with increasing heating time, indicating that DKP formation from Gly_2 (i.e., $Gly_2 \rightarrow DKP + H_2O$) was promoted with increasing heating time. This trend indicates that cyclization of Gly_2 (i.e., DKP formation) followed the formation of linear peptides on montmorillonite. DKP formation from amino acid monomers is thermodynamically and kinetically favorable at high temperatures and in dry conditions because the reaction is dehydration (Bujdak and Rode, 1996). Thus, DKP was condensed on montmorillonite because of the dehydration promoted under continuous dry condition.

Some studies have often concluded that DKP formation is a dead-end for peptide elongation (Steinberg and Bada, 1981; Basiuk et al., 1990). These experiments were performed under continuous thermal dry conditions. On the other hand, Nagayama et al. (1990) heated Gly monomer with DKP under hydrothermal conditions, and observed that amount of Gly_3 was increased with heating time. They explained that DKP formation was an important intermediate step to provide the internal free energy necessary to form additional peptide bonds, and that the peptide was elongated via the opening of the DKP ring and subsequent bonding to another amino group. The DKP molecule has two peptide bonds, and can oligomerize more easily than Gly_2 (Lambert, 2008). In this study, production of the linear tripeptide (Gly_3) on montmorillonite was limited with increasing heating time under dry conditions. Since the DKP ring has a thermally stable molecular structure, it would not easily open under high temperature and dry conditions. Therefore, DKP must be a dead-end for peptide elongation under continuous dry condition. However, peptides could be elongated to more than trimer via hydrolysis of DKP (i.e., $DKP + H_2O \rightarrow Gly_2$) if small amount of water exists.

Based on the HPLC chromatogram, Gly_4, Gly_5, and Gly_6 might be formed on

montmorillonite after heating for 336 h under dry conditions (Fig.4-5). As previously described, small amount of water was contained in montmorillonite, and it was remained after heating for 336 h under dry conditions. The water would help to hydrolyze to open the DKP rings and elongate peptides under thermal conditions.

4.1.5 Peptide formation on montmorillonite under wet conditions

Gly on montmorillonite interlayers was remained under wet conditions more than dry conditions after heating for 336 h. In other words, the reactivity of Gly on montmorillonite interlayers under wet conditions was lower than under dry conditions. The rate of dehydration is controlled by the water content of the system.

Gly was hardly decreased without montmoriilonite, while 26.7 % of Gly adsorbed on montmorillonite disappeared after heated for 336 h, probably via hydrolysis.

A small amount of short peptides (<1 %) can form under simulated hydrothermal conditions without a catalyst (Imai et al., 1999; Cleaves et al., 2009; Lemke et al., 2009). My results are in agreement with those reports: 0.7 % of Gly changed into peptides under wet conditions in the absence of montmorillonite. Most of the peptide formed on montmorillonite under dry conditions was DKP, while it was 59 % under the wet condition. Figure 4-4b shows how the ratio of products (Gly_2/DKP) changed with heating time under the wet condition. The product ratio of Gly_2/DKP increased with heating time, suggesting that the rate of cyclization of Gly_2 (i.e., $Gly_2 \rightarrow DKP + H_2O$) was controlled by excess water content.

On the other hand, no peptides were formed in the wet system with montmorillonite. As mentioned above, peptide formation is likely to be promoted through adsorption on the Lewis base sites of montmorillonite. The Lewis base site is formed through

dehydration of Si−OH and Al−OH groups. However, extra water molecules are usually coordinated to the aluminum atoms when excess water is present (Rimola et al., 2007). Thus, there should be less electrophilic defect sites under wet conditions than under dry conditions. Therefore, the peptide formation reaction may not be enhanced on montmorillonite under wet conditions because of hard dehydration.

In addition, hydrolysis of peptides would be also promoted on mineral surface in presence of excess water (Marshall-Bowman et al., 2010; Schreiner et al., 2011).

Lowing concentration of peptides on montmorillonite would be caused because of the rapid peptide hydrolysis. Although water is needed to open the DKP rings, excessive water promotes hydrolysis of DKP rather than opening of the rings. Thus, continuous wet condition is likely to inadequate to condense and elongate peptides.

It has been observed that peptide elongation is promoted under the conditions of dry–wet cycling in the presence of clay minerals (Lahav et al., 1978; Bujdak and Rode, 1999a). Bujdak and Rode (1999a) heated Gly_2 with some clay minerals under dry–wet cycling conditions at 80 °C in the open system, and observed linear peptide (Gly_4) formation. They explained that linear peptide elongation from DKP was dependent on the water activity, and dry–wet cycling, i.e., dehydration–hydration, provided the conditions of high water activity. In this study, dry–wet conditions were investigated to observe the behavior of DKP formed on montmorillonite heated at 150 °C. Water added was not escaped because of the closed system of the experiment. After addition of water to the system, the amount of Gly remaining on the montmorillonite considerably decreased (Table 3-1) and most of DKP was decomposed (Table 3-2). However, long peptides, such as Gly_3 and Gly_4, were not observed then, indicating evaporation process is essential for peptide elongation. My result reveals that continuous dry conditions and

clay minerals are needed to form intensely peptides. However, continuous dry conditions are inadequate to elongate peptides. A small amount of water must be needed to form long peptides, however, excessive water decreases the rate of peptide formation because of decomposition of the newly formed peptides.

4.1.6 Is seafloor sedimentary environments suitable for peptide elongation?

This study demonstrates that peptide formation was promoted via adsorption of amino acids on the montmorillonite under thermal condition and clay minerals would play an essential role to form peptides in natural inorganic systems. Various types of clay minerals are formed by hydrothermal alteration, and are abundantly deposited on and around the seafloor and subareal hydrothermal vent fields (e.g., Percival and Ames, 1993; Buatier and Karpoff, 1995). Thus, sedimentary conditions around hydrothermal vents are likely suitable for peptide formations.

However, peptide formation was not observed on montmorillonite under the wet condition, probably because the peptides were easily hydrolyzed and decomposed at the studied temperature. Environments required for the chemical evolution of life were likely where dehydration could occur, such as deep sediments rather than the seafloor and subareal hydrothermal systems.

As previously introduced, some studies found that high pressure was effective for elongating peptide chains under dry conditions (Ohara et al., 2007; Otake et al., 2011; Furukawa et al., 2012). Ohara et al. (2007) found that Gly_{10} was formed under high pressure (5−100 MPa) and temperature (150 °C) using simulated conditions that mimicked deep sediment beneath the seafloor where high dry condition can be normally obtained. Furukawa et al. (2011) reported that peptides of Val were synthesized under

the high temperature and pressure conditions. It is noteworthy that they observed amino acids were oligomerized under thermally dry conditions without catalysts such as clay minerals. Temperature in present seafloor sediment with usual thermal gradient becomes 150 °C at depths of 3−5 km and the porosity of argillaceous sediment remarkably decreases at deeper than 3 km (Perrier and Quiblier, 1974), estimating that deep seafloor environments must be dehydration condition and suitable to condense long peptides.

Subseafloor argillaceous sediments act as a semipermeable membrane; inorganic salts and organic molecules are filtrated and condensed, although volatile components such as H_2O, CO_2 and H_2S can pass through the membrane (Hanor, 1979). Thus, amino acids would be easily concentrated to promote the polymerization in such sediments in association with diagenetic dehydration at increasing temperature and pressure. Figure 4-6 shows a schematic model of peptide formation in deep seafloor argillaceous sediment. First, amino acids are dissolved and condensed in seawater (amino acid soup formation). The amino acids were originated endogenous (hydrothermal, impact shock, and sunlight) and/or exogenous (transportation by meteorites, comets, and asteroid). The concentration of amino acids in the primitive ocean are estimated to be between 3×10^{-4} and 0.2 M (Cleaves et al., 2009), although this is still a matter of debate. Second, amino acids adsorbed on clay minerals were deposited, buried, and finally oligomerized on the clay minerals under increasing temperature and pressure.

My result supported that a suitable environment for promoting the precursors necessary for the chemical evolution of life must include conditions suitable for dehydration such as that found in deep sediment rather than at or near the surface on the primitive Earth and/or ocean bottom.

4.2 Thermal condensation of diastereoisomeric Ala-DKP on olivine surface

4.2.1 Ala-DKP formation on olivine surface

As mentioned above, DKP formation is dehydration reaction and thermodynamically favorable under high temperature conditions. A small amount of DKP was formed when only DL-Ala is heated, however, DKP formation is remarkably promoted when heated with olivine. Olivine is thermodynamically favorable for hydration reaction (Kitadai et al., 2011), thus DKP formation (i.e., dehydration reaction) must be promoted on the olivine surface. The olivine hydration is called "serpentinization", and the Gibbs free energy is positive at even room temperature. The reaction is represented by the following equation:

$$3(Fe, Mg)_2\ SiO_4 + 2SiO_2 + 4H_2O \rightarrow 2Mg_3Si_2O_5(OH)_4$$

The DKP was not detected after heating for 8 days without water when the olivine coexisted. Although olivine would be good catalyst for DKP formation, a small amount of water is needed, probably because it plays a role to break strong bonds of the Ala crystals.

Asaduzzaman et al. (2014) explained that amino acids were strongly adsorbed via formation of an ionic bonding between positively charged Mg atom in olivine surface and negatively charged carboxyl group ($-COO^-$) of amino acid. Therefore, Ala would be activated by the adsorption on olivine surface and easily changed to DKP.

4.2.2 deastereoisomeric excess of Ala-DKP on olivine surface

The values of *deastereomeric excess* (de) of the DKP were defined following the

formula shown in below:

$$de\ (\%) = \frac{[cis\ \mathrm{DKP}] - [trans\ \mathrm{DKP}]}{[cis\ \mathrm{DKP}] + [trans\ \mathrm{DKP}]} \times 100$$

Positive *de* means excess of *cis* DKP. The *de* of DKP formed from the reaction without olivine heating for 8 days was +7.3 %. On the contrast, it was +16.3 % when reacted with olivine. Thus, the *de* of DKP was considerably increased when reacted with olivine. Olivine would be not only an efficient catalyst to promote the DKP formation but also a determining factor on the selectivity of diastereoisomeric DKP. It was reported that reactivity of the *cis*-DKP would be slightly higher than *trans*-DKP, and that the *de* gradually decreased with increasing reaction time (Naraoka and Harada, 1986). Based on a NMR study, *cis*-DKP is considered to have a boat type ring, and *trans*-DKP has a planar type ring (Fig. 4-7, Egami and Kakuta, 1973). The planar DKP is more labile than the boat type DKP, since amide bonds in the planar DKP were strained (Egami and Kakuta, 1973). When a mixture solution of liner *cis*-Ala_2 and *trans*-Ala_2 heated with montmorillonite for 5 days at 85 °C, the yield of *cis*-DKP was twice higher than that of *trans*-DKP after 5 days (Bujdak et al., 2006). The yield of DKP was significantly decreased, and the same trend was observed when the mixture heated without montmorillonite. They estimated that the configurations of molecules of liner *cis*-Ala_2 would favor the intramolecular interactions between nucleophilic amino group and electrophilic carboxyl group (i.e., peptide bond formation). In other words, *cis*-dipeptides are more rapidly cyclized to DKP than *trans*-dipeptide. Other some studies also observed the intense formation of *cis*-DKP (Saetia et al., 1993; Plasson et al., 2011), estimating that *cis*-DKP and peptides could be accumulated during thermally

abiotic peptide formation. Therefore, the selective accumulation of *cis*-DKP might be important as the first step of homogeneous peptides and protein formation on the primitive Earth.

4.3 Stabilities of amino acids under hydrothermal conditions

4.3.1 Release of amino acids from sediment to hydrothermal solution

When seafloor sediment was heated with NaCl solution at 150 °C for 24–240 h, DCAA and DFAA were rapidly released into the solution after starting the reaction, and the concentration of DCAA was consistently higher than that of DFAA (Fig. 3-8). It was revealed that >99 % amino acids in seafloor sediment collected around hydrothermal vent in Okinawa Trough were biogenic polymers (Fuchida et al., in press). Therefore, amino acids could be initially released from the sediment to the solution as DCAA.

Figure 4-8 shows changes in the percentage of DCAA to THAAs in the solution when heated at 150 °C. As explained above, most of DCAA was Glu (>50 %) in the solution heated at 150 and 200 °C. It was revealed that Glu is easily changed to stable cyclic structure under hydrothermal conditions: pyroglutamic acid (Pyro-Glu) (Fig. 4-9) (Povoledo and Vallentyne, 1964), and hard to distinguish between combined-Glu and Pyro-Glu. Therefore, Glu is excluded from this discussion here.

The percentage of DCAA to THAA in the solution at 150 °C was 65.2 % after 24 h and decreased to 52.9 % until 96 h. The decrease of DCAA ratio indicates that DCAA were immediately hydrolyzed to DFAA after released from the sediment. After that, however, the ratio of DCAA was gradually increased with increasing time and was 71.0 % after 240 h, indicating that the hydrolysis rate of DCAA was lower than the decomposition rate of DFAA. It was recognized that peptides and proteins are more labile than amino

acid monomers and rapidly hydrolyzed to monomers under hydrothermal conditions (Qian et al., 1993). However, this study documents that DCAA are more stable than DFAA under hydrothermal condition.

The ratio of DCAA to THAA in the solution at 100 °C was 90.2 % after 240 h, estimating that hydrolysis of DCAA was significantly slow at 100 °C. On the other hand, at 200 °C, the percentage was decreased to 57.7 % after heating for 240 h (Fig. 4-10). Shock (1992) suggested that the stability of peptide bond was increased at high temperature condition, and they concluded that peptide, polypeptides and proteins could survive under hydrothermal conditions because hydrolysis rate was slow. However, in this study, the DCAA ratio decreased with increasing temperature. Thus, my result suggests that the hydrolysis rate increases with increasing temperature, most of DCAA changes to DFAA at the higher temperature condition.

Figure 4-11 shows the compositions (Mol %) of DCAA in the solution heated after 240 h at 100, 150, and 200 °C versus that of THAA in the initial sediment. The compositions of DCAA after heating for 240 h at 150 and 200 °C were different compared with that of THAA in the initial sediment; a number of thermally labile amino acids such as Asp, Thr, Ser and Arg were decreased (Fig. 4-11b and c). Therefore, biopolymers released from the sediment were thermally altered and compositions might be changed over 150 °C.

On the other hand, the composition of DCAA in the solution heated at 100 °C was very similar with that of THAA in the initial sediment (Fig. 4-11a). The positive correlation between DCAA and THAA indicates that biopolymers released from the sediment would be hardly altered in the hydrothermal solution at 100 °C. Thus, low temperature hydrothermal condition (<100 °C) must be suitable to release and stabilize biopolymers

fixed in the sediment without thermal alteration and hydrolysis.

4.3.2 Thermal stabilities of amino acids under hydrothermal condition

When four types of α-amino acids (Asp, Ser, Leu, and Ala) heated under a hydrothermal condition (200 °C, 50 atm) for 47 h, Ser and Asp were rapidly decomposed while Ala was relatively stable (Andersson et al., 2000). Kohara et al. (1997) evaluated the stabilities of Asp, Glu, Gly, Ala, Val, and Leu under hydrothermal conditions at 200−350 °C for 2 h. Ser and Asp disappeared at 250 °C, while Gly, Ala and Val remained at 300 °C. Therefore, the stability of amino acids depends on their functional group. In this study, neutral amino acids, especially non-polar amino acids (Gly, Ala, Val, Ile, Leu, and Phe), remained as DCAA after heating for 240 h at 150 and 200 °C, except for Glu compared with the other amino acids.

In addition, low levels of thermally labile amino acids such as Asp, Thr, Ser, and Arg remained in the solution after heating for 240 h at 200 °C. The stabilities of these labile amino acids would be enhanced through combination with inorganic materials under hydrothermal conditions (Kawahata and Ishizuka, 1993; Ito et al., 2006). Amorphous silica, dissolved in high concentrations in hydrothermal fluids (Von Damm et al., 1985), would protect labile amino acids such as Ser from thermal decomposition. Ito et al. (2009) observed that the stability of Ser increased in simulated hydrothermal fluids when heated with siliceous ooze collected from offshore Shimokita, Japan. This behavior was likely because bonding between the hydroxyl group of Ser in proteins and silica increased the stability of Ser (Hecky et al., 1973; Chandru et al., 2013).

Metal ions, dissolved abundantly in hydrothermal solutions, would also stabilize amino acids via complexation. When six amino acids (Asp, Ser, Pro, Gly, Ala, and Val)

were heated with metal ions (Cu^{2+}, Fe^{2+}, and Mn^{2+}) in a hydrothermal flow reactor at 200−250 °C for 120 min, stabilities of these amino acids increased compared to the same reaction carried out without the metal ions (Chandru et al., 2013). Most of dissolved organic molecules (>90 %) combined with metal ions in hydrothermal fluid (Sander et al., 2007), estimating that amino acids would be also combined with metal ions and stabilized in hydrothermal fluids. Much amount of metal ions and silica would be contained in the reacted solution of this study, because these were easily released from sediment to solution under increasing temperature (e.g., Cruse and Seewald, 2001). Thus, the combinations with metal ions and silica are important to survive labile amino acids in hydrothermal solution.

Thermal stability of amino acids increased via adsorption on mineral surfaces (Kawahata and Ishizuka, 1993; Ito et al., 2006; Klevenz et al., 2010). When natural seafloor sediments containing abundant amounts of amino acids were heated under hydrothermal condition at 300 °C for 240 h, no amino acids were detected in the solution but they were detected in the sediment (Ito et al., 2009). In particular, thermally unstable amino acids (Asp, Thr, and Ser) remained in the sediments. In this study also, these labile amino acids remained in the sediment after heating at 150 °C for 240 h. Thus, coexisting mineral components must also important factor controlling amino acid distribution under hydrothermal conditions.

4.3.3 Chemical evolution in hydrothermal systems

As previously mentioned, some researchers found the formation of short peptides under simulated hydrothermal conditions, and estimated that peptides and proteins were formed in the hydrothermal systems on primitive Earth (Imai et al., 1999; Cleaves et al.,

2009; Lemke et al., 2009; Sakata et al., 2010). However, the formation rates are very small and the concentrations of amino acids and peptides decrease because of rapid hydrolysis (Qian et al., 1993; Marshall-Bowman et al., 2010). Therefore, the hydrothermal conditions are unfavorable to form and condense long peptide chains and proteins.

Nakazawa (2008) estimated that biopolymers were not formed under hydrothermal but in deep seafloor, these were released to ocean by hydrothermal fluid flows, and life precursors were formed and evolved in the ocean. My result supported that dehydration conditions are more suitable to oligomerize amino acids (i.e., peptide and protein formations) than hydration conditions. In next stage of the chemical evolution, it is necessary to survive the peptides and proteins released from seafloor sediments in hydrothermal solutions. This study found that DCAA and DFAA were easily released from sediment to hydrothermal solution, and DCAA are more stable than DFAA under hydrothermal conditions. This finding suggests that amino acids can be existed and survived as combined form (peptides and proteins) in hydrothermal fluids when amino acids initially released from the sediment to the solution as DCAA.

Ito et al. (2006) also evaluated the release process and stability of amino acids in natural sediment, and observed that the concentration of amino acids released to hydrothermal solution was the highest at 150 °C when seafloor sediment was heated with NaCl solution at 100−300 °C for 240 h. They, based on this observation, concluded that the upper temperature limit for the stable presence of amino acids probably lay between 150 and 200 °C. In this study, however, the concentrations of DCAA and DFAA after 240 h were the highest at 100 °C and decreased with increasing temperature. In addition, most of amino acids (>90 %) were combined form at 100 °C, while most of

those were hydrolyzed at 200 °C. Thus, low temperature conditions (< 100 °C) were required to survive DCAA without hydrolysis and thermal alteration.

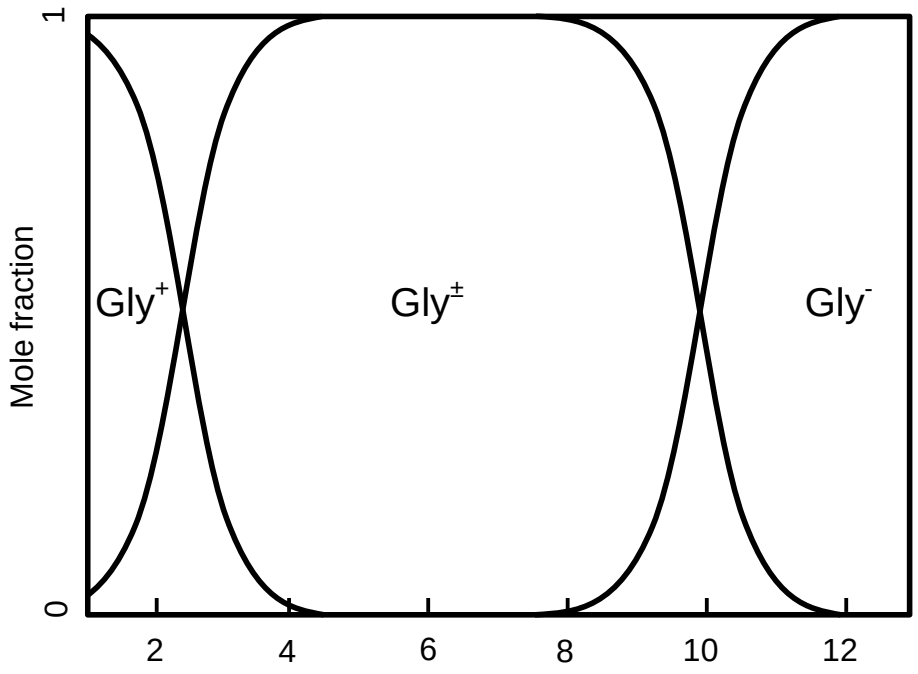


Fig. 4-1 Changes in the ionic state of Gly as a function of pH (25 °C) (Sakata et al., 2010)

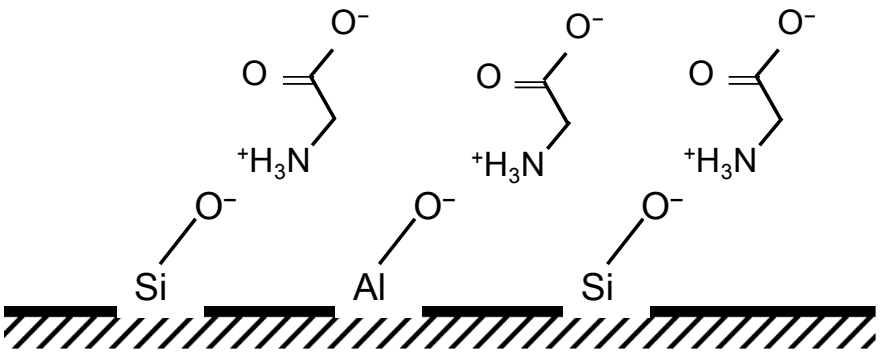


Fig. 4-2 Adsorption model of Gly in the interlayer of montmorillonite via hydrogen bonds

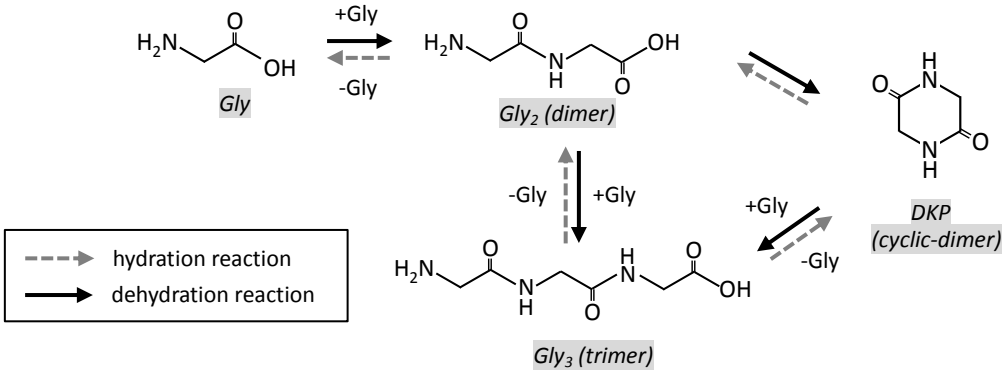


Fig. 4-3 Reaction pathway of oligomerization of Gly

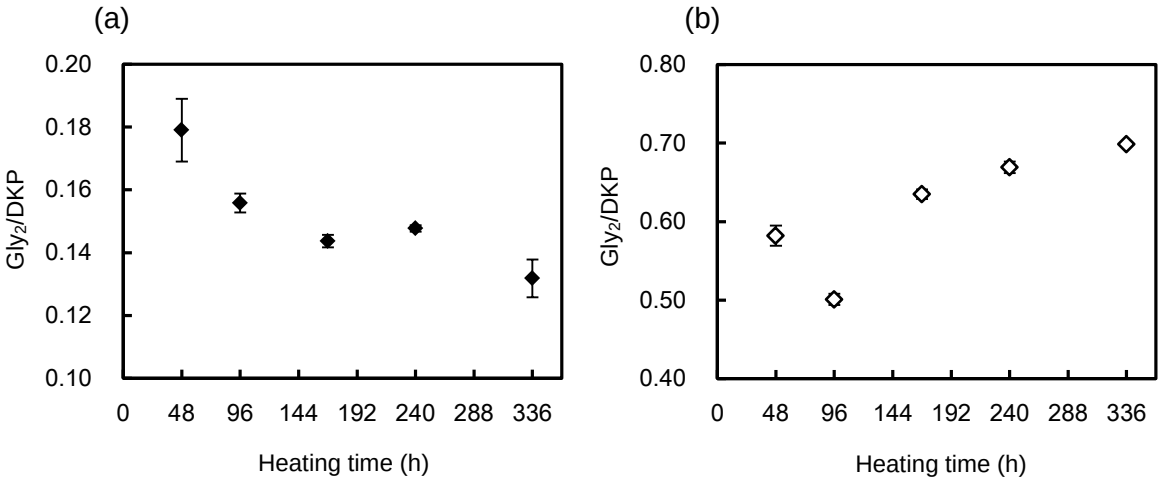


Fig. 4-4 Changes in the ratio of (a) Gly_2/DKP on montmorillonite with heating under dry conditions and (b) Gly_2/DKP without montmorillonite under wet conditions

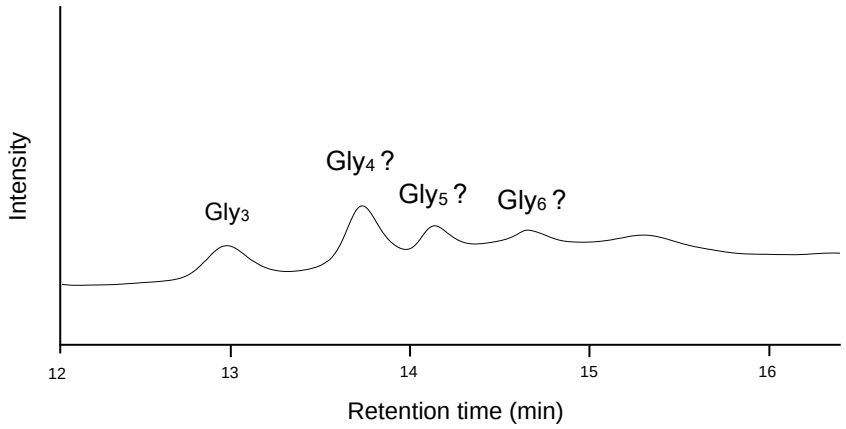


Fig. 4-5 The HPLC chromatogram of the montmorillonite sample after heating at 150 °C for 336 h under dry conditions, extended between 12 and 16 min

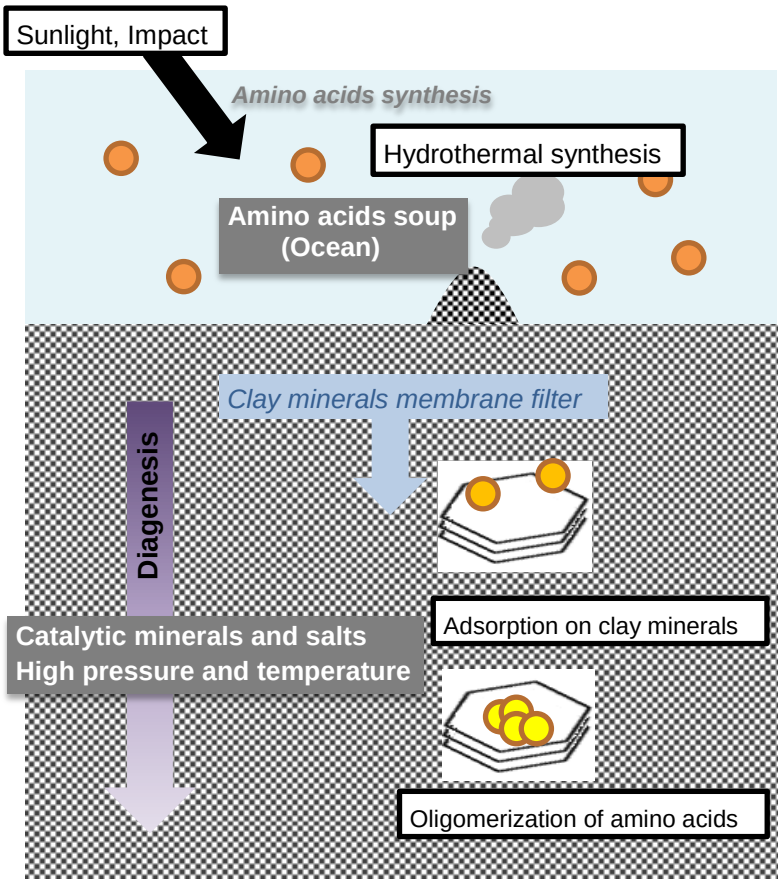


Fig. 4-6 Peptide formation model in deep seafloor sediment

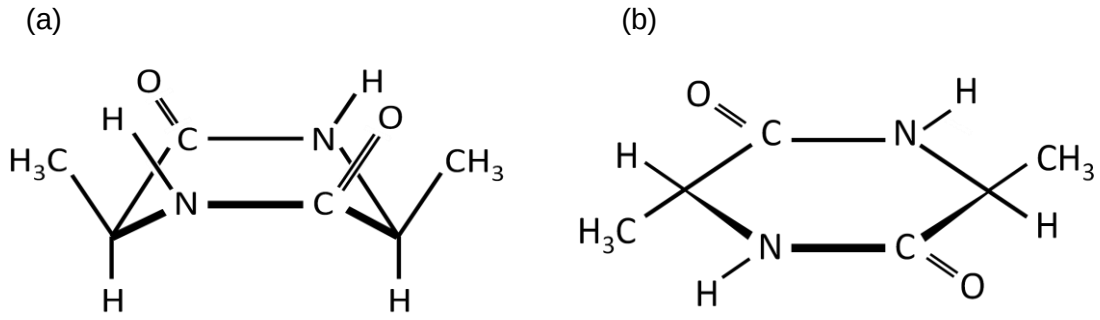


Fig. 4-7 Conformation model of (a) *cis*-DKP and (b) *trans*-DKP (Egami and Kakuta, 1973)

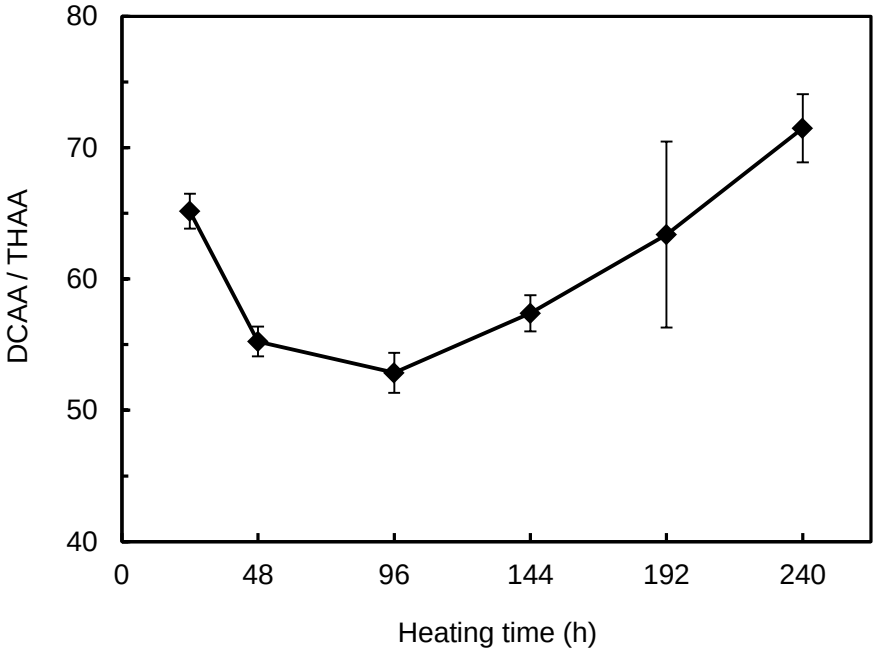
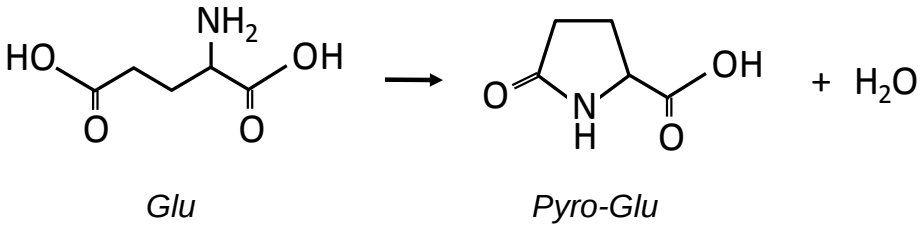


Fig. 4-8 Changes in the percentages of DCAA to THAA in the hydrothermal solutions after heating at 150 °C for 240 h



Glu *Pyro-Glu*

Fig. 4-9 Cyclization model of Glu at high temperature condition

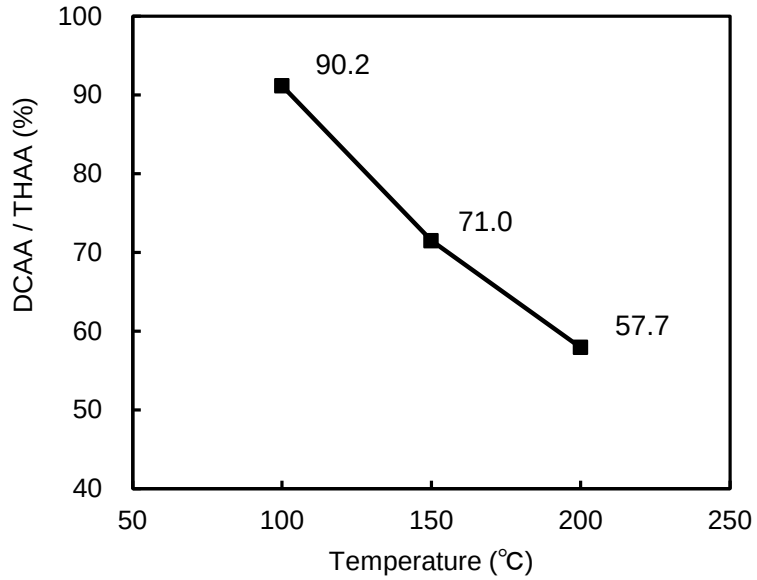


Fig. 4-10 Changes in the percentages of DCAA to THAA in the hydrothermal solutions heated at 100–200 °C for 240 h

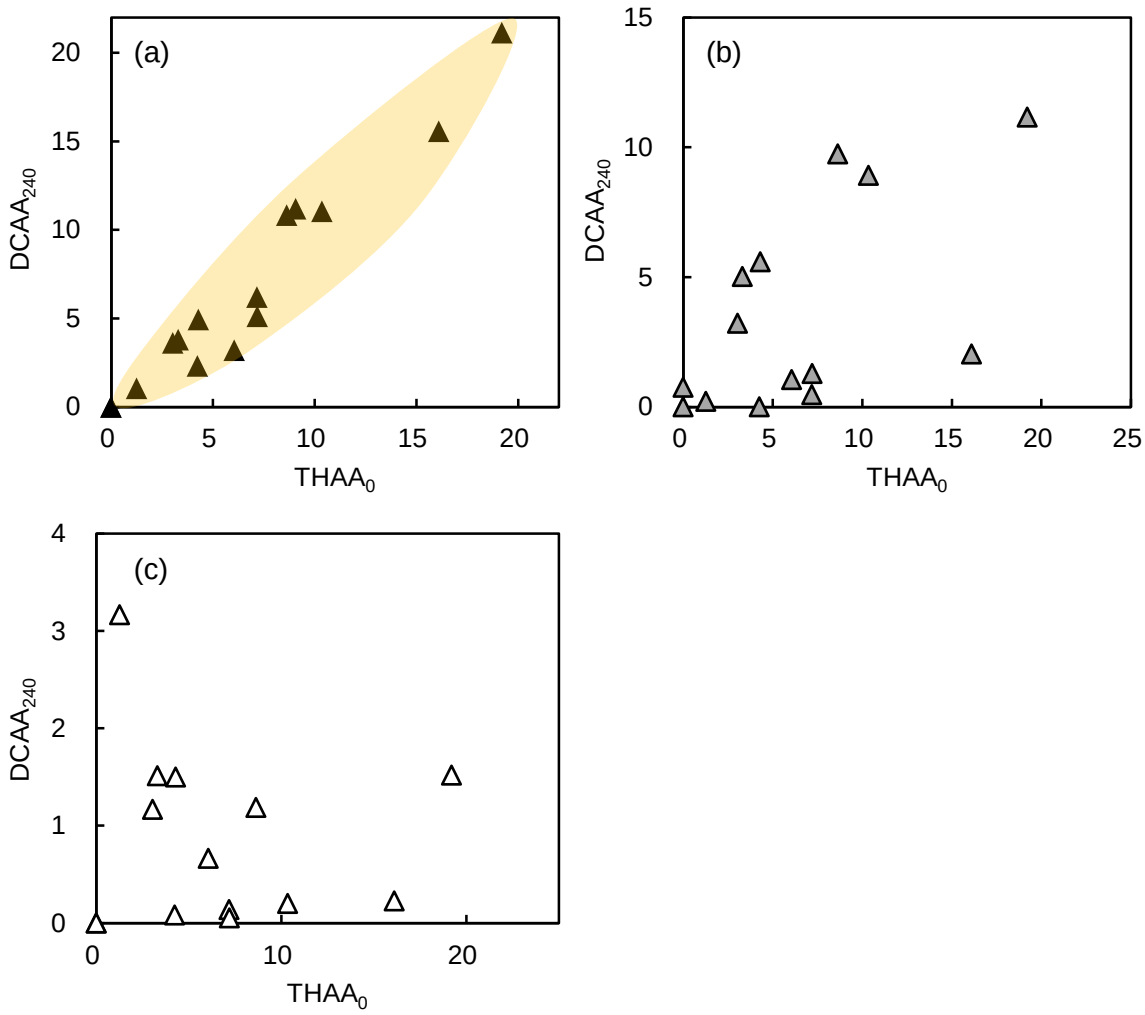


Fig. 4-11 Compositions (Mol %) of DCAA in the solution heated after 240 h at (a) 100, (b) 150, and (c) 200 °C versus composition of THAA in the initial sediment

5. Conclusions

To estimate the favorable conditions for chemical evolution of life on the primitive Earth, three different experiments were conducted in this study. These results were summarized as below;

(1) *Oligomerization of amino acids on clay mineral surface*

Gly was easily adsorbed on the interlayer of montmorillonite and its reactivity increased after the adsorption. The yield of Gly peptides that were formed on montmorillonite was higher when the reaction occurred under dry, rather than wet, conditions and DKP was the most abundant product. Excess water shifted the equilibrium towards hydrolysis of the peptides, and hydrolysis of peptides would be promoted on montmorillonite under wet condition. Thus, deep seafloor, where dehydration easily occurs, is more appropriate condition to form and condense oligomer of amino acids than the seafloor surface.

Some researchers reported that high pressure was effective for elongating peptide chains under dry condition without catalyst such as clay minerals, and estimated that peptides and proteins were condensed in deep seafloor sediment during diagenesis (Ohara et al. 2007; Otake et al., 2011; Furukawa et al., 2012). My result supports the hypothesis, and suggested that clay minerals, as catalyst of peptide formation, must be essential factors to condense peptides on the primitive earth.

(2) *Stereoisomeric selectivity of peptides on olivine surface*

DKP formation from DL-Ala was promoted on olivine surface at 120 °C. Hydration

reaction of olivine is thermodynamically favorable, thus DKP formation (i.e., dehydration reaction) was promoted on the olivine surface.

In addition, the *de* of DKP was considerably increased when reacted with olivine. Olivine would be not only an efficient catalyst to promote the DKP formation but also a determing factor on the selectivity of diastereoisomeric DKP. Thus, serpentinization of ultramafic rock would have a deep relationship to DKP formation and distribution of asymmetric stereoisomer of peptides on the primitive Earth.

(3) *Release and stability of biopolymers under hydrothermal conditions*

When seafloor sediment concentrating amino acids was heated for 240 h at 100–200 °C, amino acids were rapidly released from the sediment to the solution. Most of amino acids dissolved in the solution were combined form and the composition was very similar with that of THAA in the initial sediment when heated at 100°C. On the other hand, at 150 and 200 °C, DCAA was hydrolyzed and the compositions of DCAA changed significantly different compared with that of THAA in the initial sediment. Nakazawa (2008) estimated that biopolymers were not formed under hydrothermal systems but in deep seafloor sediments, and released from sediments to ocean via hydrothermal fluid flows, followed by the formation and evolution of life precursors in the ocean. My result supported the hypothesis; amino acids can be existed and survived as combined form in low temperature hydrothermal fluids when amino acids initially released from the sediment to the solution as DCAA (i.e., peptides and proteins). Thus, low temperature hydrothermal condition (<100 °C) must be important to dissolve and condense long peptides and protein formed in deep seafloor as a step of chemical evolution of life on the primitive Earth.

Based on these findings, the early stage of chemical evolution of biopolymers on the primitive Earth was estimated as follows: 1) Amino acids abiotically formed were accumulated in the seafloor sediment via adsorption mineral surface, and then these were oligomerized during diagenesis, 2) homogeneous peptides and proteins were selectively formed on the mineral surface during the oligomerization step, and 3) the biopolymers including peptides and proteins were released from the sediment to ocean by low temperature hydrothermal venting. The conclusions of this study strongly support the relationship between minerals and the life on the primitive Earth, and thus the terrigenous origins of the biopolymers of the Earth's life.

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